

**THE CONTROL OF ARACHIDONIC ACID TURNOVER IN  
RELATION TO PROSTAGLANDIN PRODUCTION BY THE  
GUINEA-PIG UTERUS**

**by**

**Sophie Johanna Norman**

**Thesis presented for the degree of**

**Doctor of Philosophy**

**University of Edinburgh**

**1995**



**In accordance with the requirements of regulation 3.4.7 this thesis has been composed by myself and the work presented herein is my own.**

**Sophie Johanna Norman**



*With much love and thanks for all their love and  
support over the years, I dedicate this thesis to my Mum and Dad,  
Christiane and Ronnie Norman.*

## ACKNOWLEDGEMENTS

Many, many thanks to my supervisor, Dr. N. L. Poyser, for all his encouragement, ideas and sense of humour throughout this project. In particular I wish to thank him for his patience and advice during the preparation of this thesis. I also very much enjoyed the “rugby song choruses” at Fimbush!

My project was supported by a SERC studentship.

A big thank you to Mrs. Lorna Turnbull, “the best radioimmunoassayer in the world”, for her invaluable technical assistance.

I would also like to thank Mrs. Jean Hunter and all the staff of the Animal House for the wonderful care of the guinea-pigs.

To all my friends, a big hug for all those words of wisdom shared over a few “Gordons & Tonic” and x<sup>99</sup> pints of “Addlestones”.

Good luck to James, my brother, - hope you have many more journeys around the world!

Lastly, but by no means least, a very big thank you to Franky Schneider for all his support (and the computer and printer!), and especially for his patience during the writing up of this thesis. “Vill mols merci, mein leiwen” and “Long live Luxembourg, that great nation of the EU!!”

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## GLOSSARY

AA	arachidonic acid
ACLS	acyl-CoA:lysophospholipid acyltransferase
ACS	acyl-CoA synthetase
ADP	adenosine-5'-diphosphate
ARA	aristolochic acid
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
bTP-1	bovine trophoblast protein-1
$[Ca^{2+}]_i$	intracellular calcium
cAMP	cyclic adenosine 5'-monophosphate
CDP	cytidine-5'-diphosphate
CoA	coenzyme A
CTP	cytidine-5'-triphosphate
DAG	1,2-diacylglycerol
DARS	donkey anti-rabbit serum
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
hCG	human chorionic gonadotrophin
HMB	p-hydroxymercuribenzoic acid
3 $\beta$ -HSD	3 $\beta$ -hydroxysteroid dehydrogenase-5,4-isomerase
IP <sub>3</sub>	inositol-1,4,5-triphosphate
LH	luteinizing hormone
LPC	lysophosphatidylcholine
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NRS	normal rabbit serum
oTP-1	ovine trophoblast protein -1
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	prostaglandin

PGDH	prostaglandin dehydrogenase
PGH synthase	prostaglandin endoperoxide synthase
PI	phosphatidylinositol
PIP <sub>2</sub>	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA <sub>2</sub>	phospholipase A <sub>2</sub>
PLAP	phospholipase A <sub>2</sub> -activating protein
PLC	phospholipase C
PMA	phorbol-12-myristate 13-acetate
PPi	pyrophosphate
PS	phosphatidylserine
QUIN	quinacrine
RNA	ribonucleic acid
THM	thimerosal
TFP	trifluoperazine
TMB-8	3,4,5-trimethylbenzoic acid, 8-(dimethylamino)octyl ester hydrochloride
TX	thromboxane

## ABSTRACT

Prostaglandin (PG)  $F_{2\alpha}$  is the uterine luteolytic hormone in the guinea-pig (Horton and Poyser, 1976). Oestradiol acting on a progesterone-primed uterus appears to be the physiological stimulus for increased  $PGF_{2\alpha}$  production by the guinea-pig uterus (particularly by the endometrium) towards the end of the cycle (Blatchley and Poyser, 1974; Poyser, 1983b; Poyser, 1993). Arachidonic acid (AA) is the precursor of PGs. Phospholipids appear to be the source of AA for PG synthesis in the guinea-pig uterus (Leaver and Poyser, 1981). A specific stimulation of the mechanisms involved in the uptake of AA into guinea-pig endometrium (particularly into phospholipids) has been demonstrated to occur towards the end of the oestrous cycle (Ning, Leaver and Poyser, 1983). The three enzymes acyl-CoA synthetase (ACS), acyl-CoA:lysophospholipid acyltransferase (ACLS) and phospholipase ( $PLA_2$ ) control AA turnover via reacylation and deacylation of glycerophospholipids and, consequently, may play an important role in controlling the amount of free AA available for conversion to  $PGF_{2\alpha}$ . If an increase in the activities of ACS and ACLS were to occur towards the end of the oestrous cycle in the guinea-pig endometrium, this could explain the increased incorporation of AA into endometrial phospholipids observed on day 15 of the cycle. Although an increase in the rate of AA uptake may be beneficial in providing a faster turnover of AA for PG synthesis, such an increase may actually limit PG synthesis, since, following its release by  $PLA_2$ , free AA would be reacylated at a faster rate. Indeed, this could be of importance during early pregnancy, when uterine  $PGF_{2\alpha}$  output must remain low so that luteal function is maintained. Thus, PG release may not only be controlled by  $PLA_2$ , but also by the reacylating enzymes ACS and ACLS in stimulated tissues. Therefore, the role of ACS, ACLS and  $PLA_2$  in relation to PG production in the non-pregnant and pregnant guinea-pig uterus has been investigated.

ACS, ACLS and  $PLA_2$  activities were detected in guinea-pig endometrium on both day 7 (day of low  $PGF_{2\alpha}$  output) and day 15 (day of high  $PGF_{2\alpha}$  output) of the oestrous cycle and in the endometrium of ovariectomized guinea-pigs treated with oestradiol and/or progesterone.

Treatment with aristolochic acid (ARA) and quinacrine (QUIN) (PLA<sub>2</sub> inhibitors) significantly ( $P < 0.05$ ) reduced PGF<sub>2 $\alpha$</sub>  output from both day 7 and day 15 endometrium cultured for 24 h, demonstrating the crucial role that PLA<sub>2</sub> plays in the regulation of AA release for PGF<sub>2 $\alpha$</sub>  synthesis in the guinea-pig. Unexpectedly, treatment with p-hydroxymercuribenzoic acid (HMB) and thimerosal (THM) (ACS and ACLS inhibitors) also significantly ( $P < 0.05$ ) decreased PGF<sub>2 $\alpha$</sub>  output. However, during long term inhibition of ACS and ACLS, since the rate of uptake of AA into lysophospholipids will be reduced, the amount of AA appropriately placed in the *sn*-2 position of appropriate phospholipids for the action of PLA<sub>2</sub> will also be reduced. Therefore, PLA<sub>2</sub> may be indirectly inhibited by a lack of substrate.

ACS, ACLS and PLA<sub>2</sub> activities were detected in the endometrium and conceptus of early pregnant (day 15) guinea-pigs. All three enzymes were also detected in the endometrium, subplacenta, chorio-allantoic placenta, chorion and amnion of day 29 and 36 pregnant guinea-pigs. Treatment with THM and ARA of day 22, 29 and 36 pregnant guinea-pig endometrial and fetal tissues during 24 h culture suggested that the control of AA uptake is important in the subplacenta, chorio-allantoic placenta, chorion and amnion, and that PLA<sub>2</sub> appears to have an essential role in the control of PG synthesis from the endometrium, chorion and amnion of pregnant guinea-pigs.

Treatment of day 7 non-pregnant superfused guinea-pig uterus *in vitro* with HMB, THM and, surprisingly, ARA all increased PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  outputs. HMB and THM treatment also prevented the A23187-stimulated increase in PG output. In contrast, HMB and THM treatment decreased, whilst ARA treatment increased, PGF<sub>2 $\alpha$</sub>  output from day 7 guinea-pig uterine homogenates incubated for 1 h in the presence of 2  $\mu$ g/ml AA.

In summary, ACS, ACLS and PLA<sub>2</sub> activities were all detected in the endometrium of non-pregnant, and in the endometrium and fetal tissues of pregnant, guinea-pigs. The stimulus responsible for increased PLA<sub>2</sub> activity towards the end of the cycle remains obscure. The anti-luteolytic factor produced by the guinea-pig conceptus does not appear to influence the rate of AA uptake in the endometrium or

conceptus. However, the control of AA uptake in the placenta and fetal membranes seems to have a role in the tightly regulated control of PG synthesis during pregnancy.

# CHAPTER 1 : GENERAL INTRODUCTION

## Introduction to Prostaglandins

### **1:1 History of prostaglandins**

Prostaglandins were first discovered over sixty years ago by gynaecologist Raphael Kurzrok and pharmacologist Charles Lieb in 1930 who were conducting research into methods of artificial insemination. They observed that human semen caused uterine contractions in some women and relaxation in others. Experiments carried out *in vitro* on isolated human uterine strips resulted in similar findings and led to the confusing observation that a single uterine tissue sample could either contract or relax when treated with different semen samples. Two independent workers later described the presence in human seminal plasma of a vasodepressor and smooth muscle contracting substance which was lipid-soluble and acidic in nature (Goldblatt, 1933, 1935). It was also detected in extracts of seminal plasma in monkey, goat and sheep (von Euler, 1935, 1936). The name "prostaglandin" (PG) was established by von Euler in 1935 to describe this unknown substance which appeared to originate from the prostate gland. However in 1959, Eliasson determined that the seminal vesicles, not the prostate gland, were the source of PGs in the seminal plasma, but the name prostaglandin remained. By 1939, von Euler had concluded that PG was a lipid-soluble fatty acid most probably containing a double bond and a hydroxyl group. Due to inadequate purification and analytical techniques and the fact that PGs are present in very low concentrations in tissues, research on PGs was delayed considerably. In 1949, Bergström confirmed von Euler's findings that biological activity was due to a group of highly active lipid-soluble unsaturated fatty acids. Bergström and Sjövall (1957) went on to isolate one prostaglandin "factor" (PGF) in crystalline form using techniques such as paper and gas-liquid chromatography and countercurrent distribution. By 1960, using the technique of X-ray crystallography, they had proposed structures for PGF and PGE (Bergström and Sjövall, 1960a, b). The structures were confirmed as PGF<sub>1 $\alpha$</sub>  and PGE<sub>1</sub>, respectively, using mass spectrometry (Bergström, Ryhage, Samuelsson and Sjövall, 1963).

### **1:2 Chemistry and nomenclature of prostaglandins**

The PGs consist of a family of structurally related compounds with a wide diversity of biological actions. They are all based on the structure of "prostanoic acid" and can be generally described as 20-carbon, oxygenated, unsaturated fatty acids. They are made up of a cyclopentane ring with an upper ( $\alpha$ ) and lower ( $\omega$ ) side chain

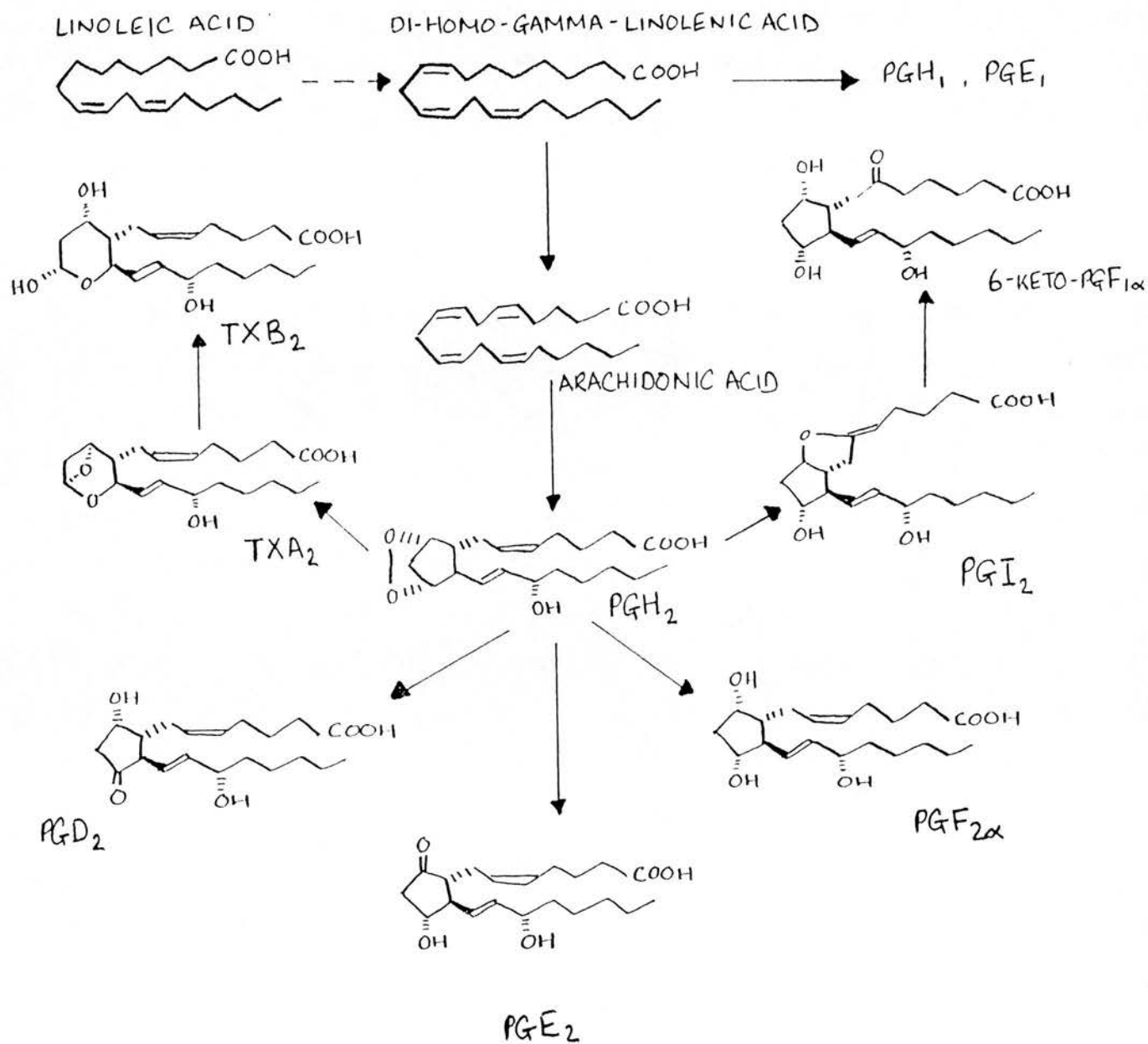
consisting of 7 and 8 carbon atoms, respectively. There are numerous structure variations which are classified into 9 different types (PGA to PGI). The letter designated to a specific PG describes differences occurring in the cyclopentane ring (with the exception of PGG<sub>2</sub> and PGH<sub>2</sub> which both have the same ring structure). Thromboxanes possess an additional oxygen atom enclosed within the ring. The PGs are also divided into 3 different "series" according to the number of double bonds in the side chains and these are represented by a subscript numeral. This degree of unsaturation of the PG is dependent on the fatty acid precursor. The fatty acid precursors of PGs of the "1", "2" and "3" series are cis-8,11,14-eicosatrienoic acid, cis-5,8,11,14-eicosatetraenoic acid and cis-5,8,11,14,17-eicosapentaenoic acid, respectively. The resulting PGs of the "1" series have a double bond in the trans 13,14 position. PGs of the "2" series have an additional double bond in the cis 5,6 position and those of the "3" series have a third double bond at the trans 17,18 position. PGs of the F group also have an additional subscript which denotes whether the carbon-9 hydroxyl group is above ( $\beta$ ) or below ( $\alpha$ ) the plane of the cyclopentane ring i.e. it describes its isomeric form. PGs have an hydroxyl group at carbon-15, and either a hydroxyl or ketone group at carbon-9 and (apart from A, B and C forms) at carbon-11. PGs of the "2" series are the major PGs found in biological systems, with the exception of primate seminal fluid where both "1" and "2" series PGs are found (Kelly, 1978). PGI<sub>2</sub> is cyclised between carbon-6 and the oxygen moiety at carbon-9. Figure 1 illustrates the chemical structures of the fatty acids used for eicosanoid synthesis, and of the PGs and thromboxanes synthesized from arachidonic acid.

### 1:3 Synthesis and metabolism of prostaglandins

PGs are not stored in tissues and therefore any increase in their tissue levels or output must be preceded by their immediate synthesis (Piper and Vane, 1971). One exception to this does exist in primate seminal fluid where PGs are synthesized by the seminal vesicles and stored in the seminal plasma (Kelly, Taylor, Hearn, Short, Martin and Marston, 1976). All cell types are capable of synthesizing PGs from fatty acids with the exception of red blood cells and sperm (Granström, 1981) and therefore PG biosynthesis can occur anywhere in the body. All naturally occurring PGs and thromboxanes are synthesized from 3 essential unsaturated fatty acids (Van Dorp, Beerthius, Ningteren and Vonkeman, 1964; Bergström, Danielsson and Samuelsson, 1964). Arachidonic acid (5,8,11,14-cis-eicosatetraenoic acid) is the most important of these fatty acids as it is the precursor of PGs of the "2" series. Formation of PGs requires that arachidonic acid be in the non-esterified form. The concentration of free arachidonic acid is very low in tissues, and therefore the release of arachidonic acid



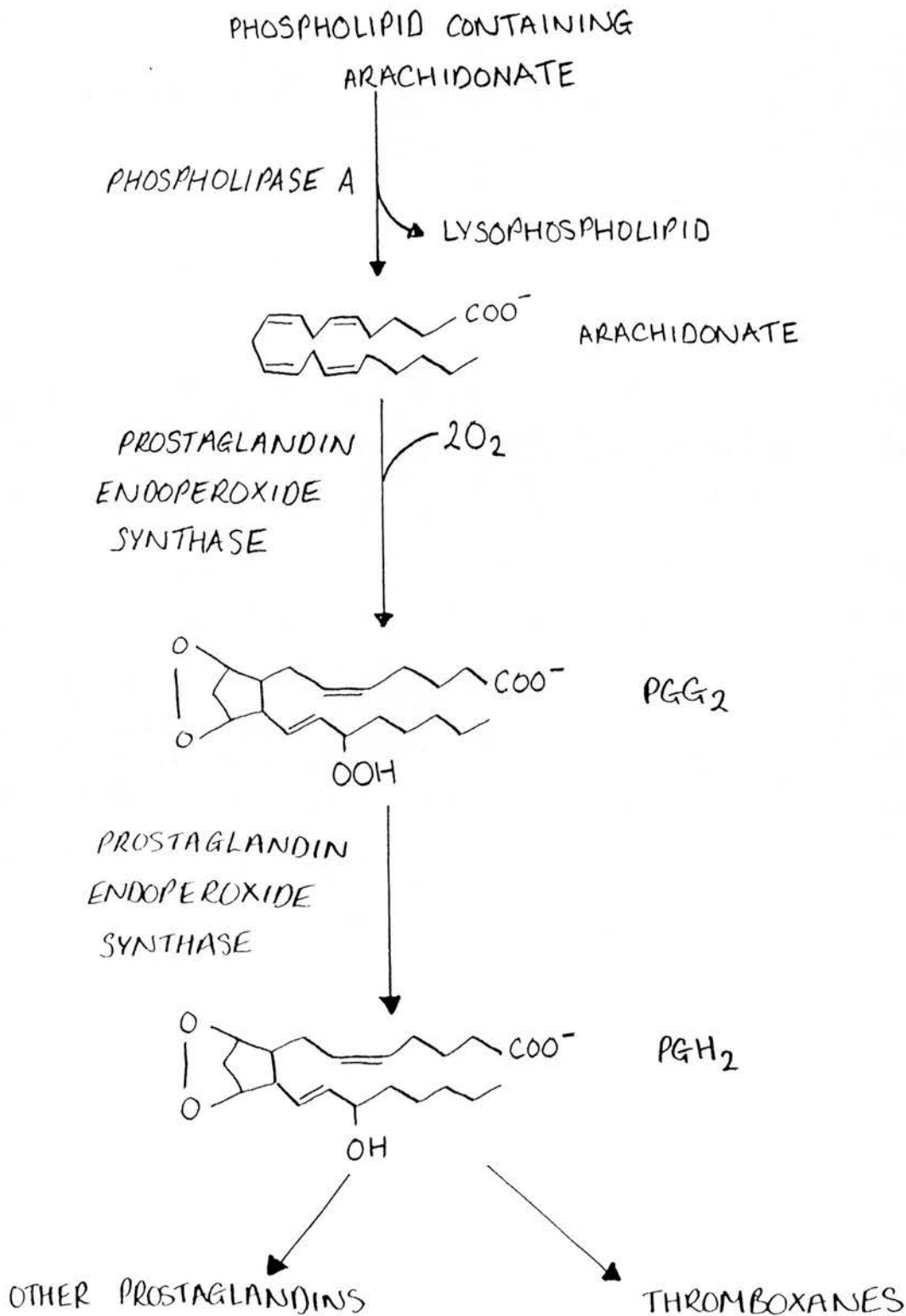
Figure 1. The chemical structures of the fatty acids used for eicosanoid synthesis, and of the prostaglandins and thromboxanes synthesized from arachidonic acid.



from some bound source is considered to be the rate-limiting step in PG synthesis. During PG synthesis, free arachidonic acid is firstly converted into the unstable (but biologically active) cyclic endoperoxide intermediates  $\text{PGG}_2$  and  $\text{PGH}_2$  (Hamberg, Svensson, Wakabayashi and Samuelsson, 1974) by the action of the enzyme prostaglandin endoperoxide (PGH) synthase. PGH synthase has two activities, namely a fatty acid cyclo-oxygenase which converts arachidonic acid to the cyclic endoperoxide, 15-hydroxyperoxide compound ( $\text{PGG}_2$ ) by the insertion of two  $\text{O}_2$  molecules at carbons 11 and 15, and a prostaglandin hydroperoxidase which then rapidly converts the  $\text{PGG}_2$  into its 15-hydroxy analogue,  $\text{PGH}_2$ . Five other enzymes with isomerase or reductase activities convert  $\text{PGH}_2$  into the specific PGs and thromboxanes (Figure 2). Which of these compounds is formed depends upon the tissue and the enzymes it possesses (Samuelsson, Goldyne, Granström, Hamberg, Hammarström and Malmsten, 1978). For example, in platelets (Hamberg, Svensson and Samuelsson, 1975) thromboxane ( $\text{TX}$ )  $\text{A}_2$  is the major compound formed, but in aortic tissue (Gryglewski, Bunting, Moncada, Flower and Vane, 1976)  $\text{PGI}_2$  is the major product. The half-life of  $\text{PGG}_2$  and  $\text{PGH}_2$  in aqueous medium at  $37^\circ\text{C}$  is approximately 5 min (Hamberg *et al.*, 1974) and the two endoperoxides may be converted to  $\text{PGE}_2$ ,  $\text{PGD}_2$ ,  $\text{PGF}_{2\alpha}$ ,  $\text{PGI}_2$  and  $\text{TXA}_2$ . Action of an isomerase enzyme on  $\text{PGH}_2$  results in  $\text{PGE}_2$ , whilst a reduction step would yield  $\text{PGF}_{2\alpha}$ . In acidic conditions  $\text{PGE}_2$  dehydrates to form  $\text{PGA}_2$  which can then be isomerised enzymatically into  $\text{PGC}_2$  and then  $\text{PGB}_2$  (Jones, Cammock and Horton, 1972). 19-Hydroxylated derivatives of  $\text{PGE}_2$ ,  $\text{PGF}_{2\alpha}$ ,  $\text{PGA}_2$  and  $\text{PGB}_2$  have also been detected.  $\text{PGI}_2$  synthase converts  $\text{PGH}_2$  to the labile but biologically active  $\text{PGI}_2$ . The  $\text{TXA}_2$  synthase enzyme catalyses the conversion of  $\text{PGH}_2$  to  $\text{TXA}_2$  (Hammarström and Falardeau, 1977). Both  $\text{PGI}_2$  (half-life 3-5 min) and  $\text{TXA}_2$  (half-life 30 s) are unstable in body fluids and rapidly form 6-keto- $\text{PGF}_{1\alpha}$  and  $\text{TXB}_2$  respectively, with a subsequent large loss in biological activity.  $\text{PGD}_2$ , an isomer of  $\text{PGE}_2$  (Granström, Lands and Samuelsson, 1968) is also formed from  $\text{PGH}_2$ .

PGs are metabolised at carbon-15 by oxidation of the secondary alcohol group into a ketone, which requires  $\text{NAD}^+$  as a co-factor. These 15-keto-prostaglandin metabolites (especially those of the F series) retain most of the biological activity of the parent compound. However subsequent reduction of the 13,14-double bond results in a much greater loss of activity, and these 13,14-dihydro-15-keto-prostaglandin compounds formed are the main circulating metabolites of  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  in the plasma. They are often present in a 20-fold greater concentration than the parent compound. Most tissues are capable of metabolising PGs, but this is often at quite a low rate. However some tissues, such as the lungs, have a very high PG

Figure 2. The reactions involved in the conversion of arachidonic acid to prostaglandins and thromboxanes.



metabolic activity (Lands, 1979; Samuelsson, Granström, Green, Hamberg and Hammarström, 1975). Between 95-99% of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  present in the circulation is metabolised by a single passage through the lungs (Ferriera and Vane, 1967). The inactivation of PGs is considered as a "two-stage" mechanism, involving an initial uptake step followed by intracellular metabolism by an  $\text{NAD}^+$ -dependent 15-hydroxyprostaglandin dehydrogenase. Bito, Baroody and Reitz (1977) suggested that the delay in the transit time of both  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  through the pulmonary circulation was due to intracellular internalisation prior to metabolism.  $\text{PGA}_2$  escapes metabolism when passing through the lungs even though it is a good substrate for the metabolising enzymes (Horton and Jones, 1969) indicating that  $\text{PGA}_2$  is not a good substrate for the uptake process (Bito *et al.*, 1977). Since  $\text{PGI}_2$  and  $\text{PGD}_2$  are also poor substrates for this uptake mechanism, they can act as circulating hormones, whilst  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  (that are both readily internalised) can only act locally. Further metabolism of the main circulating metabolites of PGs to dinor and tetranor compounds by  $\beta$ -oxidation, followed by  $\omega$ -oxidation to the 20-alcohols or carboxylic acids occurs in the liver. Hydroxylation at the 19-position can also occur, and  $\text{PGE}$  compounds can be converted to  $\text{PGF}$  derivatives. For these reasons, a number of urinary metabolites may be derived from any one PG, the main metabolite produced being dependent upon the species (Horton, 1972; Samuelsson *et al.*, 1975).

## **The Roles of Prostaglandins in Reproductive Processes**

### **1:4 Luteolysis in the non-pregnant animal**

Luteolysis (luteal regression), defined as the loss of luteal function consists of two stages; first, there is a decreased secretion of progesterone (functional luteolysis) followed by a loss of luteal tissue (structural luteolysis) (Niswender, Juengel, McGuire, Belfiore and Wiltbank, 1994). The influence of the uterus in controlling the process of luteolysis was first reported by Loeb in 1923 when he found that hysterectomy in the guinea-pig resulted in the maintenance of corpora luteal function prolonging the oestrous cycle from 15-17 to 60-120 days. In later studies, Loeb (1927) observed that the maturation of follicles was not dependent on the presence of the uterus. However, removal of unregressed corpora lutea lead to a new ovulation suggesting that cyclic changes occurring in the hysterectomized guinea-pig were due to the persistence of the corpora lutea. Loeb proposed that the guinea-pig uterus, in particular the mucosa, produced an internal secretion which acted specifically on the corpus luteum leading to its regression; he named this secretion "uterine luteolytic factor". Subsequently, removal of the uterus resulting in the prolongation of luteal

function was demonstrated in other species, including the cow and sheep (Wiltbank and Casida, 1956; Anderson, Neal and Melampy, 1962), pig (Spies, Zimmerman, Self and Casida, 1958), horse (Ginther and First, 1971) and the pseudo-pregnant rat (Bradbury, 1937), rabbit (Asdell and Hammond, 1933), mouse (Critser, Rutledge and French, 1980) and hamster (Caldwell, Mazer and Wright, 1967). However, ovarian cyclicity remained unaffected by hysterectomy in the dog (Cheval, 1934), ferret (Deanesly and Parkes, 1933) and the monkey (Burford and Diddle, 1936). In the human, hysterectomy or the congenital absence of the uterus, fallopian tubes or vagina has no effect on cyclic luteal function (Whitelaw, 1958; Doyle, Barclay, Duncan and Kirkton, 1971; Fraser, Baird, Hobson, Michie and Hunter, 1973). Specific destruction of the endometrial layer of the uterus was demonstrated to be responsible for prolongation of the oestrous cycle in the pig (Anderson, Butcher and Melampy, 1961) and guinea-pig (Butcher, Chu and Melampy, 1962) which strengthened the original proposal of Loeb (1927) that a luteolytic factor originated from the uterus and controlled luteal function and therefore oestrous cycle length. However this mechanism did not apply to all species.

### **1:5 Local nature of action of uterine luteolytic factor**

The local nature of action of the luteolytic factor was demonstrated in the guinea-pig when unilateral hysterectomy resulted in normal corpora luteal regression on the unoperated side of the uterus but luteal maintenance on the operated side (Fischer, 1965; Bland and Donovan, 1966). This effect has also been shown in the sheep (Moor and Rowson, 1966a). In both intact and unilaterally hysterectomized heifers (where the retained uterine horn was next to the ovary containing the corpora lutea), oxytocin treatment from days 3-8 shortened the oestrous cycles. However, cycles were lengthened where the retained uterine horn was contralateral to the ovary (Ginther, Woody, Mahajan, Janakiraman and Casida, 1967). The evidence suggests that the action of the luteolytic factor is limited to the ovary ipsilateral to the uterine horn from which it originates.

### **1:6 Evidence indicating $\text{PGF}_{2\alpha}$ is the uterine luteolytic hormone**

In 1968, Ducharme, Weeks and Montgomery demonstrated that  $\text{PGF}_{2\alpha}$  was a potent venoconstrictor. A short time later, it was proposed that luteolysis could be due to a reduction in ovarian blood flow and that the luteolytic factor was possibly a venoconstricting substance produced by the uterus. When infused into rats on days 5 and 6 of pseudopregnancy,  $\text{PGF}_{2\alpha}$  caused both structural and functional luteal regression compared to saline-treated animals (Pharriss and Wyndgarden, 1969).

Numerous studies that followed demonstrated that  $\text{PGF}_{2\alpha}$  administration shortened the lifespan of the corpus luteum in the guinea-pig (Blatchley and Donovan, 1969), rabbit (Gutknecht, Cornette and Pharriss, 1969; Gutknecht, Duncan and Wyndgarden, 1972), sheep (McCracken, Glew and Scaramuzzi, 1970; Chamley, Buckmaster, Cain, Cerini, Cerini, Cumming and Goding, 1972), monkey (Kirton, Pharriss and Forbes, 1970), cow (Louis, Hafs and Morrow, 1972; Lauderdale, 1972; Rowson, Tervit and Brand, 1972), horse (Douglas and Ginther, 1972; Noden, Oxender and Hafs, 1974), pig (Gleeson, 1974; Guthrie and Polge, 1976; Lindloff, Holtz, Elsaesser, Kreikenbaum and Smidt, 1976; Moeljono, Bazer and Thatcher, 1976), goat (Ott, Nelson and Hixon, 1980), hamster (Labhsetwar, 1971), mouse (Labhsetwar, 1972) and rat (Chatterjee, 1973). In order to clarify that  $\text{PGF}_{2\alpha}$  was the uterine luteolytic factor, it was necessary to show that it was present in the uterine venous blood prior to luteal regression. Increasing concentrations of  $\text{PGF}_{2\alpha}$  were observed in sheep uterine venous blood on days 14, 15 and 16 of the oestrous cycle (Bland, Horton and Poyser, 1971) and were in the same range as those which had been previously demonstrated to be capable of decreasing progesterone levels (McCracken *et al.*, 1970). This observation was confirmed by McCracken, Carlson, Glew, Goding, Baird, Green and Samuelsson (1972), Thorburn, Cox, Currie, Restall and Schneider (1972), and Fitzpatrick and Sharma (1973) when increasing  $\text{PGF}_{2\alpha}$  concentrations were measured in the uterine venous blood of sheep just prior to the occurrence of luteolysis.  $\text{PGF}_{2\alpha}$  was also detected in the utero-ovarian vein of the guinea-pig in higher concentrations from day 11 of the oestrous cycle, with a peak occurring on day 15 concurrent with a sharp decline in progesterone levels (Blatchley, Donovan, Horton and Poyser, 1972; Earthy, Bishop and Flack, 1975). An increase in the release of  $\text{PGF}_{2\alpha}$  from the uterus at the time of progesterone decline has also been demonstrated in the pig (Gleeson and Thorburn, 1973; Gleeson, Thorburn and Cox, 1974; Moeljono, Thatcher, Bazer, Frank, Owens and Wilcox, 1977; Hunter and Poyser, 1982), goat (Homeida and Cooke, 1982) and cow (Nancarrow, Buckmaster, Chamley, Cox, Cumming, Cummins, Drinan, Findlay, Goding, Restall, Schneider and Thorburn, 1973; Shemesh and Hansel, 1975). In pseudopregnant rabbits, a rapid decline in progesterone was found to be directly related to an increase in the  $\text{PGF}_{2\alpha}$  concentrations in the uterine venous plasma (Lytton and Poyser, 1982).

The introduction of glass beads into the guinea-pig uterus caused a shortening of the oestrous cycle (Donovan and Traczyk, 1962; Bland and Donovan, 1966). Poyser, Horton, Thompson and Los (1971) demonstrated that such a distension of the uterine horns resulted in a large release of  $\text{PGF}_{2\alpha}$ . These observations suggested that  $\text{PGF}_{2\alpha}$  was released due to the distension of the uterus, and then had a luteolytic



effect on the ovary. Bland and Donovan (1965) demonstrated that if only one uterine horn was distended in the guinea-pig, then only the corpora lutea of the ipsilateral ovary regressed prematurely. Similarly, in sheep where only one uterine horn had been distended with an intra-uterine device, only the corpora lutea of the ipsilateral ovary regressed prematurely. At autopsy, these corpora lutea were found to be smaller than normal, confirming that luteolysis had occurred (Ginther, Pope and Casida, 1966). Spilman and Duby (1972) demonstrated increased levels of endometrial  $\text{PGF}_{2\alpha}$  in close proximity to an intra-uterine insert in sheep, and peripheral plasma levels of  $\text{PGF}$  were also elevated in rats and hamsters possessing such a device (Saksena, Lau and Castracane, 1973). Blatchley, Donovan and Poyser (1976) recorded that in animals fitted with intra-uterine beads, an increased  $\text{PGF}_{2\alpha}$  concentration was evident in the utero-ovarian venous plasma.

In particular cases the corpus luteum is not always maintained for its normal duration. Such short luteal phases have been studied in the cow, occurring at puberty (Gonzales-Padilla, Niswender and Wiltbank, 1975) and during the transition from postpartum anoestrus to cyclicity (Odde, Wood, Kiracofe, McKee and Kittock, 1980). Evidence suggests that premature luteolysis occurs as a result of an early release of uterine  $\text{PGF}_{2\alpha}$  (Zollers, Garverick, Youngquist, Ottobre, Silcox, Copelin and Smith, 1991)

Further evidence demonstrating that  $\text{PGF}_{2\alpha}$  is the uterine luteolytic hormone in numerous species includes active immunization against  $\text{PGF}_{2\alpha}$ , and also the administration of indomethacin:-

i. Effect of immunization against  $\text{PGF}_{2\alpha}$  on luteolysis

Following immunization against  $\text{PGF}_{2\alpha}$ , antibodies are produced that neutralise the activity of endogenous  $\text{PGF}_{2\alpha}$  present in the circulation. Active immunization against  $\text{PGF}_{2\alpha}$  lengthened the oestrous cycle of the sheep (Scaramuzzi, Baird, Wheeler and Land, 1973) and the guinea-pig (Horton and Poyser, 1974; Hildebrandt-Stark, Marcus, Yoshinaga, Behrman and Greep, 1975). A good correlation was found between the extent to which the oestrous cycle was extended and the titre of the  $\text{PGF}_{2\alpha}$  antibody present in individual guinea-pigs (Horton and Poyser, 1974). Measurement of plasma progesterone concentrations indicated that, following immunization against  $\text{PGF}_{2\alpha}$ , the corpora lutea were maintained in a functional secretory state (Poyser and Horton, 1975). Similar results were found in the cow and sheep (Fairclough, Smith and McGowan, 1981). Also, in post-partum cows immunization procedures against  $\text{PGF}_{2\alpha}$  resulted in luteal maintenance of greater than 39 days (Copelin, Smith, Keisler and Garverick, 1989).

## ii. Effect of indomethacin administration on luteolysis

Indomethacin is a potent inhibitor of PG synthesis by blocking the cyclo-oxygenase component of the PGH synthase complex (Vane, 1971), and has been shown to inhibit PG production by guinea-pig uterine homogenates (Poyser, 1972). Therefore, administration of indomethacin to a species where  $\text{PGF}_{2\alpha}$  is the uterine luteolytic hormone, such as the guinea-pig, should result in the inhibition of PG synthesis and therefore prolong luteal function. Yet, when indomethacin was administered to guinea-pigs orally or subcutaneously, oestrous cycles were not prolonged for more than 3-4 days (Marley, 1972; Horton and Poyser, 1973). However, the administration of indomethacin in the form of a slow-release preparation within the lumen of the guinea-pig uterus did greatly prolong luteal function (Horton and Poyser, 1973) and maintained high progesterone levels (Poyser and Horton, 1975). Concerning other species, subcutaneous administration of indomethacin resulted in extended length of pseudopregnancy comparable to that due to hysterectomy in the pseudopregnant rabbit (O'Grady, Caldwell, Auletta and Speroff, 1972). Similar results have been reported in the pseudopregnant mouse (Critser, Rutledge and French, 1981), rat and hamster (Lau, Saksena and Chang, 1975). Subcutaneously administered indomethacin was demonstrated to delay the pulsatile appearance of  $\text{PGF}_{2\alpha}$  metabolite (13,14-dihydro-15-keto- $\text{PGF}_{2\alpha}$ ) and luteolysis in the goat (Cooke and Homeida, 1985), and also to prolong luteal maintenance in the pig. However, in hysterectomized gilts, luteal function was unaffected by indomethacin treatment indicating that the source of luteolytic  $\text{PGF}_{2\alpha}$  in the pig is the uterus (Kraeling, Rampacek and Kiser, 1981). In the sheep and cow, intra-uterine infusions of indomethacin prolonged luteal function (Lewis and Warren, 1975, 1977a) and also inhibited oestrogen-induced luteolysis (Lewis and Warren, 1974, 1977b). There is controversy whether indomethacin is able to act systemically or needs to be given locally to block uterine  $\text{PGF}_{2\alpha}$  synthesis in sheep. Oral or parenteral administration of various PGH synthase inhibitors failed to prolong the oestrous cycle in one study (Mellin and Busch, 1976), but subcutaneous indomethacin treatment from day 7 of the cycle onwards did prevent loss of luteal function, as detected in maintained high plasma progesterone levels, in 3 out of 5 ewes. Following slaughter on day 23, normal corpora lutea were observed suggesting that indomethacin administered systemically is able to prevent luteolysis (Kann and Lacroix, 1982). This idea is strengthened by previous work by Spilman and Duby (1972) who demonstrated that subcutaneous administration of indomethacin abolished previously observed increases in  $\text{PGF}_{2\alpha}$  concentrations in the uterine vein of ewes fitted with an intra-uterine device. In normally cycling Rhesus monkeys, indomethacin blocked oestradiol-induced luteolysis and was associated with a significant increase in



peripheral plasma progesterone concentration (Auletta, Caldwell and Speroff, 1976, 1978, Auletta, Agins and Scommegna, 1978) suggesting that PGs may play an important role in luteal function in the monkey. In women,  $\text{PGF}_{2\alpha}$  infused intravenously on day 21 of the oestrous cycle did result in a sharp drop in progesterone concentrations (Lehmann, Peters, Breckwoldt and Bettendorf, 1972). However, other studies found that such treatment did not affect the length of the luteal phase or the pattern of steroid secretion in normally cycling women (LeMaire and Shapiro, 1972; Jewelewicz, Cantor, Dryenfurth, Warren and Van de Wiele, 1972) or in pregnant women prior to abortion (Speroff, Caldwell, Brock, Anderson and Hobbins, 1972), thus it is unlikely that  $\text{PGF}_{2\alpha}$  has a luteolytic function in women.

### **1:7 Mechanism by which $\text{PGF}_{2\alpha}$ causes luteal regression**

The evidence presented has clearly shown that  $\text{PGF}_{2\alpha}$  is the uterine luteolytic hormone in several species. At the onset of luteal regression, serum progesterone concentrations decrease (Diekman, O'Callaghan, Nett and Niswender, 1978a; Baird, 1984) followed by a loss of luteal weight (Diekman *et al.*, 1978b; Braden, Gamboni and Niswender, 1988). Structural changes in the corpus luteum include accumulation of lipid droplets in the cytoplasm of luteal cells, degeneration of capillaries and an increase in the number of primary lysosomes (Nett, McClellan and Niswender, 1976). As luteolysis progresses there is an eventual reduction in the number of steroidogenic luteal cells (Farin, Moeller, Sawyer, Gamboni and Niswender, 1986; Braden *et al.*, 1988). Evidently, the process of luteal regression comprises complex changes within the corpus luteum, so how exactly does  $\text{PGF}_{2\alpha}$  cause luteolysis?

#### **1:7:1 $\text{PGF}_{2\alpha}$ and ovarian blood flow**

DuCharme *et al.* (1968) demonstrated that  $\text{PGF}_{2\alpha}$  possessed potent vasoconstrictor properties leading to the hypothesis that  $\text{PGF}_{2\alpha}$  could cause a reduction in blood flow to the ovary, perhaps even to the corpora lutea specifically, resulting in luteal regression due to anoxia (Pharriss and Wyndgarden, 1969; Pharriss, Cornette and Gutnecht, 1970). Indeed, the corpus luteum is a highly vascularized gland receiving over 80 % of the ovarian blood supply (Nett *et al.*, 1976). Several studies followed to investigate changes in ovarian blood flow during the oestrous cycle. Sheep luteal tissue was shown to possess a high blood flow rate particularly at times when functional corpora lutea were present (Thorburn and Hales, 1972) and the administration of  $\text{PGF}_{2\alpha}$  caused a rapid decrease in luteal blood flow (Nett *et al.*, 1976). Niswender, Moor, Akbar, Nett and Diekman (1975) demonstrated that luteal and ovarian blood flow rates decreased around the time of luteal regression leading to the proposition that blood flow to the ovary, particularly to the corpora lutea, played

an important role in the control of luteolysis (Niswender, Reimers, Diekman and Nett, 1976). This hypothesis was further supported by work on the guinea-pig using labelled microspheres which reported high luteal and ovarian blood flow rates only up until day 12 of the oestrous cycle (Hossain, Lee, Clarke and O'Shea, 1979). However, measurement of the ovarian blood flow in sheep failed to show a decrease in blood flow prior to the decline in progesterone levels associated with luteal regression (McCracken *et al.*, 1970; Baird, 1974). Later, Einer-Jensen and McCracken (1977) reported that the decrease in progesterone secretion from the corpora lutea at the time of luteolysis in the sheep occurred before a decrease in ovarian blood flow was observed, supporting the idea that vasoconstriction due to  $\text{PGF}_{2\alpha}$  was not the initial stimulus, but a consequence, of luteal regression. O'Shea, Nightingale and Chamley, (1977) suggested that the decline in ovarian blood flow observed during luteal regression was possibly due to small capillaries in the ovary becoming narrow by being blocked by cell debris from the regressing corpora lutea.

### **1:7:2 $\text{PGF}_{2\alpha}$ receptors in corpora lutea**

Specific  $\text{PGF}_{2\alpha}$  receptors have been shown to be present in the luteal cell membrane in the sheep (Powell, Hammerström and Samuelsson, 1974), cow (Rao, Carman and Gorman, 1978), rat (Wright, Luborskymoore and Behrman, 1979), mare (Kimball and Wyndgarden, 1977) and human (Powell, Hammarström, Samuelsson and Sjoberg, 1974). Presumably,  $\text{PGF}_{2\alpha}$  must bind with its luteal cell membrane receptor to induce luteolysis, and the variation in  $\text{PGF}_{2\alpha}$  receptor number and affinity during the oestrous cycle may be a mechanism of controlling the timing of luteolysis. Indeed, in the cow, an increase in the affinity of receptors in the corpus luteum results in an elevation of  $\text{PGF}_{2\alpha}$  binding, with maximum receptor affinity occurring at the time of actual luteal regression (Rao, Estergreen, Carman and Moss, 1979). In the pseudopregnant rat,  $\text{PGF}_{2\alpha}$  binding to the corpora lutea increases up to a maximum on day 7, and this is followed by a gradual decline after day 9 preceding luteolysis (Brambaifa and Schillinger, 1984). However, in the mare, variation in  $\text{PGF}_{2\alpha}$  receptor numbers was apparent but no relation between receptor number and the state of the oestrous cycle was observed (Kimball and Wyndgarden, 1977).

### **1:7:3 Role of oestradiol in luteolysis**

It has been proposed that oestradiol is required for the luteolytic action of  $\text{PGF}_{2\alpha}$  to be realized, since  $\text{PGF}_{2\alpha}$ -induced luteolysis is prevented in hysterectomized ewes where the oestrogen-producing ovarian follicles have been previously destroyed by X-irradiation (Hixon, Gengenbach and Hansel, 1975). This is further supported by the observation that luteolysis does occur in hysterectomized

ewes treated with  $\text{PGF}_{2\alpha}$ , in doses below the threshold for inducing luteolysis, in conjunction with oestradiol treatment (Gengenbach, Hixon and Hansel, 1977).

#### **1:7:4 Relevance of presence of large and small luteal cells**

In several species such as the sheep (Fitz, Mayan, Sawyer and Niswender, 1982), cow (Urseley and Leymarie, 1979) and pig (Lemon and Moir, 1977), two morphologically and functionally distinct steroidogenic cell types exist within the corpus luteum, namely small (12-22  $\mu\text{m}$  diameter) and large (>22  $\mu\text{m}$  diameter) luteal cells. Both cell types secrete progesterone although unstimulated secretion of progesterone is 5-10 times greater in the large than in the small luteal cells (Fitz *et al.*, 1982; Rodgers, O'Shea and Findlay, 1983a). However, there is a greater proportion of small cells present so that each group secretes an equivalent amount of progesterone (Rodgers, O'Shea and Findlay, 1983a).

Progesterone secretion is dependent on luteinizing hormone (LH) and cyclic adenosine 5'-monophosphate (cAMP) in small luteal cells since treatment with adenylate cyclase-activating agents, such as forskolin or cholera toxin, stimulates progesterone secretion (Hoyer, Fitz and Niswender, 1984). This is not so in large luteal cells (Niswender, Schall, Fitz, Farin and Sawyer, 1985), as treatment with forskolin or cholera toxin does not result in enhanced progesterone production even though these agents increase the occupancy of cAMP-dependent protein kinase by cAMP (Niswender and Hoyer, 1985). These findings indicate that progesterone production is probably not controlled by cAMP in large luteal cells.

The majority of  $\text{PGF}_{2\alpha}$  receptors are found in large luteal cells (Fitz *et al.*, 1982) and it was suggested that an increase in the numbers of the large type of cell towards the end of the oestrous cycle could explain the increase in sensitivity of the corpora lutea to  $\text{PGF}_{2\alpha}$  (Fitz and Sawyer, 1982). Niswender and Hoyer (1985) added the proposition that LH could achieve this increase in large luteal cells by stimulating the differentiation of small to large cell types. However, no such differentiation occurred as the number of large ovine luteal cells was not found to change during the oestrous cycle (O'Shea, Rodgers and Wright, 1986). These studies supported the previous suggestion that, during the cycle, large luteal cells increase in size (not in number), and by possessing a greater number of receptors than the small cells, are more susceptible to the action of  $\text{PGF}_{2\alpha}$ . The inhibition of secretion of progesterone by  $\text{PGF}_{2\alpha}$  has been demonstrated in large but not small ovine luteal cells (Wegner, Martinez-Zaguilan, Wise, Gillies and Hoyer, 1990). High-affinity receptors for  $\text{PGF}_{2\alpha}$  have also only been detected in large ovine luteal cells providing further evidence that the luteolytic signal initiated by  $\text{PGF}_{2\alpha}$  is mediated by the large, not small, luteal cells (Wiltbank, Guthrie, Mattson, Kater and Niswender, 1989).

## 1:7:5 Inhibition of progesterone secretion by $\text{PGF}_{2\alpha}$

### i. Synthesis of progesterone.

The substrate for the synthesis of progesterone is cholesterol. Free cholesterol is either used for steroid synthesis or is converted into cholesterol esters and stored as lipid droplets (Jefcoate, McNamara, Artemenko and Yamazaki, 1992). The enzyme, cholesterol esterase, releases cholesterol from cholesterol esters. Activity of the enzyme is regulated by the phosphorylation of two serine residues. Phosphorylation of one serine residue by cAMP-dependent protein kinase (PK) A activates the enzyme, whereas phosphorylation of the other residue by  $\text{Ca}^{2+}$  /calmodulin-dependent protein kinase prevents activation of the enzyme (Jefcoate *et al.*, 1992). The conversion of cholesterol to pregnenolone involves three proteins, adrenodoxin, adrenodoxin reductase and cytochrome P450<sub>sc</sub>. Pregnenolone is converted to progesterone by the action of  $3\beta$ -hydroxysteroid dehydrogenase-5,4-isomerase ( $3\beta$ -HSD) (Labrie, Simard, Luu-The, Belanger and Pelletier, 1992).

### ii. Actions of $\text{PGF}_{2\alpha}$

Within 15 min of  $\text{PGF}_{2\alpha}$  administration in the corpus luteum, the LH-induced increase in cAMP is prevented in the rat (Lahav, Freud and Lindner, 1976) and in the sheep (Fletcher and Niswender, 1982). LH binding to its receptor activates adenylate cyclase causing an increase in cAMP which then stimulates luteal progesterone synthesis and release. This inhibitory effect of  $\text{PGF}_{2\alpha}$  on LH-stimulated adenylate cyclase is unlikely to be directly mediated by a G-protein (McCann and Flint, 1993).

It was proposed that a reduction in cAMP levels leads to the dephosphorylation of cholesterol esterase to its inactive form therefore inhibiting progesterone synthesis (Henderson and McNatty, 1975). Following  $\text{PGF}_{2\alpha}$  administration, cholesterol esterase activity was seen to decline in the rat ovary; however, a greater effect was seen on the activity of cholesterol esterase synthetase (Behrman, MacDonald and Greep, 1971). Other studies on the corpora lutea of rat (Jordon, 1981) and cow (Pate and Condon, 1984) also reported inhibition by  $\text{PGF}_{2\alpha}$  of cAMP-induced progesterone production by inhibiting cholesterol esterase and cholesterol ester synthetase (Torday, Jefcoate and First, 1980). Also in hysterectomized guinea-pig,  $\text{PGF}_{2\alpha}$  treatment resulted in a 50 % reduction in the conversion of pregnenolone to progesterone by inhibiting the activity of cholesterol esterase (although not cholesterol ester synthetase) and  $3\beta$ -HSD (Dwyer and Church, 1979a, b). In the ovary,  $\text{PGE}_2$ -induced progesterone production is neither inhibited *in vivo* or *in vitro* by  $\text{PGF}_{2\alpha}$  (McNatty, Henderson and Sawers, 1975; Henderson, Scaramuzzi and Baird, 1977) indicating that  $\text{PGF}_{2\alpha}$  specifically antagonises LH, presumably at a point inhibiting cAMP production.

After binding to its receptor,  $\text{PGF}_{2\alpha}$  through a direct or indirect action must prevent the LH receptor from stimulating adenylate cyclase (Henderson and McNatty, 1975). This is further exemplified by the finding that administration of cAMP to rat luteal cells cultured *in vitro* overcomes the inhibition in progesterone secretion due to the action of  $\text{PGF}_{2\alpha}$  (Thomas, Dorflinger and Behrman, 1978). One possible mechanism by which  $\text{PGF}_{2\alpha}$  may prevent LH-induced cAMP production was proposed by Luborsky, Slater and Behrman (1984) whereby  $\text{PGF}_{2\alpha}$  prevents LH receptor movement and LH receptor up-regulation. Once LH binds to its receptor, LH-receptor complexes move and bind together forming "aggregates" which appear to be necessary for the activation of adenylate cyclase and therefore cAMP production. In the rat ovary, microfilaments are involved in LH-induced adenylate cyclase activity, so that  $\text{PGF}_{2\alpha}$  may have a luteolytic action directed towards the microfilaments preventing receptor mobility and redistribution (Zor, Strulovici and Lindner, 1978).

On receiving a pulse of LH, binding of LH to luteal cells increases within 2h suggesting that an up-regulation of available LH membrane receptors occurs. It is therefore also possible that  $\text{PGF}_{2\alpha}$  prevents LH binding to its receptor by simply inhibiting the availability of LH receptors in luteal cell membranes so that during luteal regression LH receptor number and LH binding steadily decrease along with a resulting decline in progesterone levels. LH binding to its receptor is reduced by  $\text{PGF}_{2\alpha}$  in the rat (Behrman, Grinwich, Hichens and MacDonald, 1978), sheep (Diekman *et al.*, 1978b) and pig (Barb, Kraeling, Rampacek and Pinkert, 1984). It has been proposed that this prevention in the appearance of LH receptors, LH binding and movement of LH-receptor complexes is due to the phosphorylation of membrane proteins by a calcium-dependent, calmodulin-mediated mechanism resulting in membrane rigidification (Riley and Carlson, 1985). In the rat, administration of  $\text{PGF}_{2\alpha}$  decreases plasma membrane fluidity and increases superoxide radical formation (Carlson, Buhr and Riley, 1989; Sawada and Carlson, 1991). Even though the number of LH receptors has been shown to decrease following  $\text{PGF}_{2\alpha}$  treatment both in rats (Hichens, Grinwich and Behrman, 1974) and sheep (Diekman *et al.*, 1978b), in the latter study the decrease in receptor numbers was not evident until after a significant decrease in progesterone concentrations had already occurred, suggesting that the initial luteolytic signal is mediated via some other mechanism.

The main antisteroidogenic effect of  $\text{PGF}_{2\alpha}$  appears to be mediated through the protein kinase C (PKC) second messenger system.  $\text{PGF}_{2\alpha}$  has been shown to activate phospholipase (PL) C via a pertussis toxin-sensitive G-protein in the sheep corpus luteum (McCann and Flint, 1993). This results in the hydrolysis of membrane



phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) to inositol-1,4,5-triphosphate (IP<sub>3</sub>) and 1,2-diacylglycerol (DAG) (Berridge, 1987). DAG increases the affinity of PKC for calcium and IP<sub>3</sub> releases calcium from intracellular stores resulting in a rise in free intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>) concentrations and the activation of PKC. The administration of PGF<sub>2</sub>α resulted in activation of PKC in ovine large luteal cells (Wiltbank, Diskin and Niswender, 1991) and in an increase in PIP<sub>2</sub> hydrolysis in luteal cells from Rhesus monkey (Houmard, Guan, Stokes and Ottobre, 1992). Pharmacological activation of PKC has also been shown to reduce progesterone production from ovine large luteal cells (Wiltbank *et al.*, 1991; Conley and Ford, 1989; Hoyer and Marion, 1989) and isolated rat luteal cells (Baum and Rosberg, 1987). The antisteroidogenic effects of PKC do not appear to act directly on steroidogenic enzymes, but do inhibit the transport of cholesterol to cytochrome P450<sub>sc</sub> (Wiltbank, Belfiore and Niswender, 1993). However, PGF<sub>2</sub>α has also been shown to decrease steady-state levels of mRNA encoding 3β-HSD (Hawkins, Belfiore, Kile and Niswender, 1993).

The pharmacological activation of PKC has no detrimental effects on cell viability in the sheep (Wiltbank *et al.*, 1991; Hoyer and Marion, 1989). There is now accumulating evidence that the second messenger system actually mediating the luteolytic effects of PGF<sub>2</sub>α is free [Ca<sup>2+</sup>]<sub>i</sub>. In most tissues maintenance of calcium homeostasis is important for basic cellular function and optimal responsiveness to hormonal stimulation. PGF<sub>2</sub>α has been shown to induce both an initial and sustained post transient increase in free [Ca<sup>2+</sup>]<sub>i</sub> in ovine (Wegner *et al.*, 1990), bovine (Alila, Corradino and Hansel, 1989) and monkey (Houmard *et al.*, 1992) luteal cells. Large sustained increases in free [Ca<sup>2+</sup>]<sub>i</sub> in myocardial cells (Buja, Hagler and Willerson, 1988) and hepatocytes (Starke, Hoek and Farber, 1986) have been shown to decrease cellular function and ultimately lead to cell death. Ovine large luteal cells cultured in the presence of A23187 died in culture since they are unable to equilibrate large increases in free [Ca<sup>2+</sup>]<sub>i</sub> (Hoyer and Marion, 1989). Since the end result of luteal regression is demise of the corpus luteum, the sustained increase in free [Ca<sup>2+</sup>]<sub>i</sub> initiated by PGF<sub>2</sub>α in large luteal cells may be one mechanism responsible for decreased secretion of progesterone and cell degeneration associated with luteolysis.

More recent studies have demonstrated that ovine large and small luteal cells differ in their regulation of intracellular calcium homeostasis. Treatments that increase free [Ca<sup>2+</sup>]<sub>i</sub> result in a decrease in progesterone secretion in large cells, but have no effect in small cells (Martinez-Zaguilan, Wegner, Gillies and Hoyer, 1994). Therefore, luteal regression at the cellular level appears to be associated with a PGF<sub>2</sub>α-induced increase and sustained elevation in free [Ca<sup>2+</sup>]<sub>i</sub>, which is observed in large, but not

small, luteal cells (Horion and Marion, 1989; Wiltbank *et al.*, 1989; Wegner *et al.*, 1990, 1991). Further work has shown that  $\text{PGF}_{2\alpha}$  releases calcium from a thapsigargin-sensitive intracellular calcium pool in ovine large luteal cells (Wegner, Martinez-Zaguilan, Gillies and Hoyer, 1994). Thapsigargin, a sesquiterpene lactone tumor promoter, specifically inhibits a  $\text{Ca}^{2+}$ -ATPase associated with the endoplasmic reticulum. By contrast, the calcium ionophore A23187 causes an increase in free  $[\text{Ca}^{2+}]_i$ , by promoting influx of  $\text{Ca}^{2+}$  in most cell types. Inhibition of secretion of progesterone by thapsigargin has been demonstrated in mixed suspensions of rat luteal cells (Pepperell and Behrman, 1990) and by A23187 in ovine large (but not small) luteal cells (Hoyer and Marion, 1989) suggesting that extracellular, as well as intracellular, calcium contributes to the  $\text{PGF}_{2\alpha}$ -induced increase in cytosolic free calcium concentrations in large luteal cells. However, in ovine large luteal cells, progesterone secretion was found to be inhibited by either a sustained increase or decrease in free  $[\text{Ca}^{2+}]_i$ , providing evidence that optimal secretion of progesterone is dependent upon a specific range of free  $[\text{Ca}^{2+}]_i$  within these cells (Wegner *et al.*, 1991). It is possible that key enzymes responsible for progesterone synthesis are not adequately stimulated at reduced  $[\text{Ca}^{2+}]_i$  concentrations, whereas sustained elevations in  $[\text{Ca}^{2+}]_i$  may inhibit secretion of progesterone due to cytotoxic effects of calcium on a variety of cellular reactions.

In summary, a reduced capacity of ovine large luteal cells to regulate  $[\text{Ca}^{2+}]_i$  may facilitate the induction of luteal regression in these cells by  $\text{PGF}_{2\alpha}$  (Martinez-Zaguilan *et al.*, 1994). Furthermore, whereas an adequate concentration of  $[\text{Ca}^{2+}]_i$  is required to support progesterone production in both large and small luteal cells, optimal progesterone production in large cells depends upon an appropriate window of  $[\text{Ca}^{2+}]_i$  (Wegner *et al.*, 1991).

It has also been reported that ovine luteal cells appear to undergo apoptosis during  $\text{PGF}_{2\alpha}$ -induced luteolysis (Sawyer, Niswender, Braden and Niswender, 1990). It is therefore possible that the antisteroidogenic actions of  $\text{PGF}_{2\alpha}$  are mediated by the activation of the PKC pathway while the luteolytic actions of  $\text{PGF}_{2\alpha}$  may result from the process of apoptosis, with the increase in concentrations of free  $[\text{Ca}^{2+}]_i$  being the signal for induction of this process (Niswender *et al.*, 1994).

### **1:8 Transfer of the uterine luteolytic factor from the uterus to the ovary**

Studies involving ligation of the vascular tissue in the area surrounding the uterus and ovary in the guinea-pig, resulted in maintenance of the corpora lutea. However, when ligation of the oviduct was carried out, no effect on luteal regression was observed indicating that uterine  $\text{PGF}_{2\alpha}$  was transferred to the ovary by some

mechanism via the vasculature (Bland and Donovan, 1969a; Fisher, 1967; Oxenreider and Day, 1967). Similar findings were observed in the rat (Clemens, Minaguchi and Meites, 1968; Butcher, Barley and Inskeep, 1969) and in the hamster (Orsini, 1968). In the sheep, bilateral ligation of the middle uterine arteries and veins resulted in luteal maintenance, although this was not observed when ligation of the arteries alone was performed (Kiracofe, Menzies, Grier and Spies, 1966; Kiracofe, Spies and Grier, 1973) indicating that an intact uterine vein was essential for luteal regression to occur. Separation of the ovarian artery from the utero-ovarian vein in the sheep was also seen to prevent luteal regression (Barrett, Blockey, Brown, Cumming, Goding, Mole and Obst, 1971) and a counter-current mechanism of transfer of  $\text{PGF}_{2\alpha}$  from the uterine vein to the ovarian artery was proposed. Del Campo and Ginther (1972) had previously reported that the ovarian arteries were always in close proximity and association, and frequently coiled around the uterine veins in the guinea-pig, rat and hamster. In the sheep and cow, Del Campo and Ginther (1973) observed that the ovarian artery appeared to follow a "torturous" path over the utero-ovarian vein, and suggested that the ovarian artery was the means by which  $\text{PGF}_{2\alpha}$  reached the ovary. Estimated amounts of labelled  $\text{PGF}_{2\alpha}$  transferred via a counter-current mechanism from the uterine vein to the ovarian artery in the sheep have been found to be 2 % (McCracken *et al.*, 1972), 0.1-0.56 % (Land, Baird and Scaramuzzi, 1976) and 0.3 % (Heap, Fleet and Hamon, 1985). This efficiency of transfer would seem very low, although, it must be noted that if the efficiency of transfer were much greater,  $\text{PGF}_{2\alpha}$  levels would increase and remain high due to the inability of the local tissues to metabolise prostaglandins. If the transfer process were 100% efficient and the metabolism nil,  $\text{PGF}_{2\alpha}$  would pass through the ovary *ad infinitum* (Horton and Poyser, 1976). Consequently, any  $\text{PGF}_{2\alpha}$  not transferred to the ovarian artery will pass into the systemic circulation and be metabolised in the lungs (Ferriera and Vane, 1967). Hixon and Hansel (1974) have also demonstrated this counter-current mechanism to occur in the cow. Alawachi, Bland and Poyser (1981) also proposed the existence of an additional pathway for  $\text{PGF}_{2\alpha}$  transfer to the ovary via the venous drainage passing alongside the oviduct, as  $\text{PGF}_{2\alpha}$  levels are higher in the oviducal vein than in the peripheral venous plasma between days 12 and 16 of the sheep oestrous cycle. A counter-current transfer of  $\text{PGF}_{2\alpha}$  between the oviducal vein and ovarian artery could also occur.

Lamond and Drost (1973) demonstrated that the interruption of the section of ovarian artery distal to the region where counter-current transfer had been reported to occur, did not disrupt the oestrous cycle in the ewe, and suggested that another mechanism of  $\text{PGF}_{2\alpha}$  transfer to the ovary from the uterus must exist. Furthermore,



the uterine and ovarian vasculature are independent in both the rabbit (Hunter and Casida, 1967) and horse (Del Campo and Ginther, 1973) supporting the view that another mechanism of transfer exists, as a counter-current mechanism cannot justify the transfer of  $\text{PGF}_{2\alpha}$  from the uterine vein to the ovarian artery in these two species. Unusual findings were reported in the pig when it was observed that hemihysterectomy still maintained bilateral luteal regression. Removal of one ovary and autotransplantation of the remaining ovary to the vacant site also had no effect on luteolysis. Since no common utero-ovarian vessels were present, and the uterine vein and ovarian artery were separated by a distance of at least 5 cm, no counter-current mechanism could occur (Harrison and Heap, 1972). A possible explanation for these findings was that  $\text{PGF}_{2\alpha}$  was acting systemically since, unlike most species, in the pig the majority of  $\text{PGF}_{2\alpha}$  passes through the lungs unchanged (Davies, Fleet, Harrison and Maule Walker, 1980). However, infusion of  $\text{PGF}_{2\alpha}$  into the jugular vein of the sow did not cause luteal regression (Kotwica, 1980) which contradicted a systemic transfer of  $\text{PGF}_{2\alpha}$ , and it was therefore proposed that the lymph system provided a mechanism of transport of  $\text{PGF}_{2\alpha}$  in view of the rich lymphatic vascularization present in the sow uterus. A counter-current transfer of  $\text{PGF}_{2\alpha}$  between the lymph vessels and the ovarian artery was also suggested to exist in the sheep (Staples, Fleet and Heap, 1982). Increased amounts of  $\text{PGF}_{2\alpha}$  were detected in the uterine lymph of non-pregnant sheep onwards from day 10 of the oestrous cycle (Abdel Rahim, Bland and Poyser, 1983; 1984a). Abdel Rahim and Bland (1985) proposed that the close association between the uterine lymphatic vessels and the ovarian artery close to the utero-ovarian vein could provide a counter-current transfer mechanism from the uterine lymph to the ovarian artery. Evidence for this theory was soon provided by Heap *et al.*, (1985) who administered  $[^3\text{H}]\text{-PGF}_{2\alpha}$  into the uterine lumen of non-pregnant sheep 7-15 days following oestrus. A peak of radioactivity was recorded in the uterine lymph vessels within 50 min. The estimated efficiency of  $\text{PGF}_{2\alpha}$  transfer from a lymphatic vessel to the ovarian artery was found to be 0.4 %; a value similar to that of the transfer from uterine vein to ovarian artery (0.3 %). Infusion of  $[^3\text{H}]\text{-PGF}_{2\alpha}$  into the lymphatic vessels themselves resulted in a higher concentration of radioactivity in the plasma from adjacent utero-ovarian veins and ovarian veins than in the peripheral plasma. The local nature of this transfer was clearly observed by the higher concentrations of  $[^3\text{H}]\text{-PGF}_{2\alpha}$  present in the ovary and corpora lutea adjacent to the site of intra-lymphatic infusion in comparison with those tissues opposite. Abdel Rahim, Bland and Poyser (1984b) demonstrated the need for this counter-current mechanism in the sheep by insertion of a glass canula into the uterine branch of the utero-ovarian vein, followed by disconnection of all other tissue between the

uterus and ovary. Luteal regression did not take place suggesting that uterine venous blood alone is not able to supply enough  $\text{PGF}_{2\alpha}$  from the uterus to the ipsilateral ovary for luteal regression to occur. This alternative method of transfer of  $\text{PGF}_{2\alpha}$  via the lymphatic system could explain how luteal regression by  $\text{PGF}_{2\alpha}$  occurs in species such as the horse (Del Campo and Ginther, 1973) where anatomical differences do not appear to allow a counter-current mechanism to operate within the vascular system alone. The collected evidence suggests that transfer of uterine  $\text{PGF}_{2\alpha}$  to the ovary for the purpose of luteal regression may be mediated by a combination of two counter-current mechanisms involving both uterine venous blood and uterine lymph, the relative importance of each being species dependent.

### **1:9 Stimulus responsible for $\text{PGF}_{2\alpha}$ synthesis and release by the uterus**

The cyclical pattern of release of  $\text{PGF}_{2\alpha}$  indicated that some form of hormonal control was probably involved in the physiological stimulus for  $\text{PGF}_{2\alpha}$  synthesis and release by the uterus. Numerous studies followed to investigate the role of the ovarian steroids on uterine  $\text{PGF}_{2\alpha}$  synthesis and release.

Subcutaneous administration of oestradiol to day 7 guinea-pigs was found to result in premature luteal regression and shortening of the oestrous cycle (Choudary and Greenwald, 1968; Bland and Donovan, 1968). This effect was abolished in hysterectomized animals showing that the uterus was involved in mediating the luteolytic action of oestradiol (Bland and Donovan, 1970). Injection of oestradiol on day 10 and 11 of the oestrous cycle of the ewe also caused early luteal regression but failed to do so in hysterectomized ewes (Stormshak, Kelley and Hawk, 1969). Bolt and Hawk (1972) failed to induce luteal regression in hysterectomized ewes in any trial with exogenous oestradiol supporting the theory that oestradiol-induced luteal regression in the guinea-pig and sheep requires the presence of the uterus. Similar findings were also reported in the cow (Eley, Thatcher and Bazer, 1979). Corpora luteal mass was reduced in cycling, pregnant and hysterectomized cows following oestradiol treatment, although progesterone levels remained high in the hysterectomized cows indicating the necessity of the uterus for the complete luteolytic effect of oestradiol to be realized (Kaltenbach, Niswender, Zimmerman and Wiltbank, 1964). An increase in oestradiol concentrations was found to occur at the time of follicular growth in the sheep (Smeaton and Robertson, 1971), and destruction of the ovarian follicles mechanically (Ginther, 1970) or by X-ray irradiation (Karsch, Noveroske, Roche, Norton and Nalbandov, 1970) prevented luteal regression in both the sheep and cow (Villa-Godoy, Ireland, Wortman, Ames, Hughes and Fogwell,

1985). The collected evidence clearly indicated that oestradiol was causing luteal regression via a response mediated by the uterus.

Blatchley *et al.* (1972) observed that oestradiol treatment of guinea-pigs on days 4 to 6 of the oestrous cycle resulted in a premature increase in the concentration of  $\text{PGF}_{2\alpha}$  in the utero-ovarian plasma on day 7. This effect was not seen in hysterectomized animals indicating that  $\text{PGF}_{2\alpha}$  originated from the uterus. In the sheep uterus autotransplanted to the neck and infused with oestradiol *in vivo*,  $\text{PGF}_{2\alpha}$  output was stimulated after 60 to 90 min (Barcikowski, Carlson, Wilson and McCracken, 1974). It was proposed that endogenous oestradiol released from the developing ovarian follicles was the physiological stimulus for uterine  $\text{PGF}_{2\alpha}$  synthesis and release. Subsequently, the oestradiol concentration present in the guinea-pig ovarian venous plasma was shown to begin to increase on days 9 to 10 of the oestrous cycle (Joshi, Watson and Labhsetwar, 1973) preceding the increase in  $\text{PGF}_{2\alpha}$  concentration which begins on day 11 of the cycle (Blatchley *et al.*, 1972; Earthy *et al.*, 1975). Similar observations were reported in the sheep (Cox, Thorburn, Currie and Restall, 1974; Thorburn, Cox, Currie, Restall and Schneider, 1973), cow (Shemesh, Ayalon and Lindner, 1972; Nancarrow *et al.*, 1973), pig (Henricks, Guthrie and Handlin, 1972; Gleeson and Thorburn, 1973; Gleeson *et al.*, 1974), pseudopregnant rat (Welschen, Osman, Dullaart, de Greef, Ulienbroek and de Jong, 1975) and pseudopregnant hamster (Shaikh, Birchall and Saksena, 1973).

A controversy arose over the exact role of ovarian oestradiol in the stimulus for uterine  $\text{PGF}_{2\alpha}$  output from the uterus in sheep when no relationship was found between oestradiol concentrations in the plasma and the initial increase in  $\text{PGF}_{2\alpha}$  release (Baird, Land, Scaramuzzi and Wheeler, 1976; Ottobre, Lewis, Thayne and Inskeep, 1980). Destruction of sheep ovarian follicles, to remove the major source of oestradiol, only delayed luteal regression (Warren, Hawk and Bolt, 1973), and passive immunization against endogenous oestradiol prevented oestrus and ovulation but not luteal regression (Fairclough, Smith and Peterson, 1976), suggesting that a rise in plasma oestradiol concentrations was not the only factor required for normal luteolysis in the sheep. Other studies in ovariectomized sheep reported that the output of  $\text{PGF}_{2\alpha}$  from the uterus was increased by oestradiol treatment following several days of exogenous or endogenous progesterone treatment (Scaramuzzi, Baird, Boyle, Land and Wheeler, 1977; Barcikowski *et al.*, 1974). In the ovariectomized sheep treated with progesterone for 7 days, a significant increase in  $\text{PGF}_{2\alpha}$  concentration in the uterine venous plasma was observed, although progesterone treatment for a further 7 days caused a decrease in  $\text{PGF}_{2\alpha}$  output from the uterus (Scaramuzzi *et al.*, 1977). Ottobre *et al.* (1980) subsequently demonstrated that early treatment of sheep

with progesterone on days 0 and 1 of the oestrous cycle advanced the occurrence of the initial increase in uterine  $\text{PGF}_{2\alpha}$  output to day 8 compared to day 12 in control untreated animals. Also, infusion of progesterone to ewes for 10 days (immediately following oestrus) resulted in a stimulation of  $\text{PGF}_{2\alpha}$  output from the uterus which peaked on days 9-10 (McCracken, 1980). These findings suggested that a period of progesterone secretion was also necessary for increased uterine  $\text{PGF}_{2\alpha}$  output and that the timing of this increase was dependent upon when plasma progesterone concentrations increased.

Blatchley and Poyser (1974) treated ovariectomized guinea-pigs with oestradiol and /or progesterone, and measured the subsequent  $\text{PGF}_{2\alpha}$  concentrations in the utero-ovarian venous plasma. Progesterone and oestradiol treatment alone resulted in  $\text{PGF}_{2\alpha}$  concentrations similar to those normally found on days 3 and 10 of the cycle, respectively. However, only 2 of the 6 animals treated with oestradiol exhibited  $\text{PGF}_{2\alpha}$  concentrations higher than those treated with progesterone alone. In animals treated with a progesterone implant for 7 days followed by 3 days simultaneous progesterone and oestradiol treatment,  $\text{PGF}_{2\alpha}$  concentrations were greatly elevated in all guinea-pigs.  $\text{PGF}_{2\alpha}$  concentrations in the uterine tissue of ovariectomized mice significantly increased after 3 days of progesterone followed by 3 days of oestradiol treatment when compared to mice treated with either steroid alone (Saksena and Lau, 1973).  $\text{PGF}_{2\alpha}$  levels in the peripheral plasma of ovariectomized ewes were also reported to rise after treatment with oestradiol following a series of progesterone injections (Caldwell, Tilson, Brock and Speroff, 1972). The evidence pointed to the theory that oestradiol acting on a progesterone-primed uterus was the physiological stimulus responsible for the output of luteolytic  $\text{PGF}_{2\alpha}$  from the uterus. However, whether a specific stimulation of  $\text{PGF}_{2\alpha}$  was occurring was not known until Antonini, Turner and Pauerstein (1976) demonstrated that  $\text{PGF}_{2\alpha}$  output from the guinea-pig uterus *in vivo* increased without any change in  $\text{PGE}_2$  output. Further work by Poyser (1983b) investigating the effect of oestradiol and progesterone treatment on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  (the stable metabolite of  $\text{PGI}_2$ ) in ovariectomized guinea-pigs found that the outputs of all three PGs were low from untreated ovariectomized guinea-pig uteri and progesterone treatment had no significant effect on any of the PG outputs. However, oestradiol treatment significantly stimulated the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  7-fold, 1.7-fold and 2.9-fold, respectively. When the uteri were primed with progesterone prior to oestradiol treatment, a further 2-fold increase in  $\text{PGF}_{2\alpha}$  output was observed, but no significant increases occurred in  $\text{PGE}_2$  or 6-keto- $\text{PGF}_{1\alpha}$  outputs when compared to PG outputs from oestradiol-only treated uteri. Oestradiol



may have a dual action in increasing uterine PG synthesizing capacity and directing synthesis toward PGF<sub>2α</sub>. The essential role of the ovarian steroids in luteolysis in the guinea-pig was demonstrated when the administration of onapristone (a progesterone antagonist) and ICI 182780 (an oestrogen antagonist) on days 11-14 of the oestrous cycle significantly decreased uterine PGF<sub>2α</sub> output from the day 15 guinea-pig uterus. Plasma progesterone levels remained elevated confirming that luteal regression had been prevented (Poyser, 1993a). Thus it appears that oestradiol acting on a progesterone primed uterus is the physiological stimulus for the increase in PGF<sub>2α</sub> synthesis and release from the guinea-pig uterus at the time of luteolysis.

The passive immunization of sheep against either oestradiol or progesterone failed to prevent complete luteolysis in either animals (Fairclough *et al.*, 1976; Fairclough, Smith, Peterson and McGowan, 1976) suggesting that oestrogen and progesterone may not be the only factors involved in the control of uterine PGF<sub>2α</sub> output in the sheep. However, a later study demonstrated that adequate progesterone exposure during the early to mid-luteal phase of the oestrous cycle is essential for the initiation of ovarian-uterine mechanisms that lead to luteolysis in ewes (Morgan, Geisert, McCann, Bazer, Ott, Mirando and Stewart, 1993).

### **1:10 Role of oxytocin in uterine PGF<sub>2α</sub> production**

The potent spasmogenic action of oxytocin was first demonstrated by Dale in 1906 when he showed that intravenously administered extracts of pituitary gland resulted in strong uterine contractions in the pregnant cat. Oxytocin was later shown to shorten the oestrous cycle in cyclic heifers when administered during the first week following oestrus (Auletta, Currie and Black, 1972; Anderson and Bowerman, 1963). This effect was shown to occur only in animals with an intact uterus (Armstrong and Hansel, 1959; Anderson, Bowerman and Melampy, 1965) indicating that oxytocin was acting via some mechanism mediated by the uterus. In unilaterally hysterectomized heifers, oxytocin treatment resulted in shortened cycles only in those animals where the remaining uterine horn was adjacent, not contralateral, to the ovary containing a corpus luteum, thus indicating a local utero-ovarian mechanism (Ginther *et al.*, 1967). The proposition then arose that oxytocin was involved in the stimulation of luteolytic uterine PGF<sub>2α</sub> synthesis and release.

Following oxytocin treatment of heifers during the early part of the oestrous cycle, an increase in uterine PGF<sub>2α</sub> output and decrease in jugular venous progesterone levels was observed (Newcomb, Booth and Rowson, 1977; Milvae and Hansel, 1980). Earlier studies had demonstrated that, in the sheep, the stimulation of PGF<sub>2α</sub> output from the uterus by an oxytocin infusion into the uterine arterial supply

was greatest after day 14 of the cycle i.e. during the luteal phase of the oestrous cycle (Roberts, Barcikowski, Wilson, Skarnes and McCracken, 1975; Wilson, Roberts and McCracken, 1974) and was unrelated to myometrial activity (Roberts and McCracken, 1976). Oxytocin was also found to stimulate  $\text{PGF}_{2\alpha}$  release from the uterus in the rat (Campos, Liggins and Seamark, 1980), rabbit (Small, Gavagan and Roberts, 1978), goat (Cooke and Homeida, 1982, 1985) and cow (Wathes, Swann, Birkett, Porter and Pickering, 1983).

The administration of oxytocin stimulates the release of  $\text{PGF}_{2\alpha}$  from the ovine uterus *in vivo* (Sharma and Fitzpatrick, 1974; Roberts *et al.*, 1975) and from ovine endometrial explants *in vitro* (Roberts, McCracken, Gavagan and Soloff, 1976; Silvia and Homanics, 1988). Immunization against oxytocin has also been found to delay luteal regression in the sheep (Flint, Mitchell and Sheldrick, 1979; Sheldrick, Mitchell and Flint, 1980; Schams, Prokoff and Barth, 1983) and goat (Cooke and Homeida, 1985). In sheep,  $\text{PGF}_{2\alpha}$  can also stimulate luteal secretion of oxytocin (Flint and Sheldrick, 1982b; Watkins and Moore, 1987; Lamsa, Kot, Eldring, Nay and McCracken, 1989). Therefore, luteal oxytocin and uterine  $\text{PGF}_{2\alpha}$  appear to exist in a positive feedback loop that may contribute to the endogenous secretion of  $\text{PGF}_{2\alpha}$  during luteal regression. Flint, Sheldrick, Theodosios and Wooding (1986) proposed that endogenous oxytocin, of luteal origin, plays an essential role in regulating uterine secretion of  $\text{PGF}_{2\alpha}$  in the sheep.

In contrast to the sheep, goat and cow, oxytocin does not appear to be involved in the release of  $\text{PGF}_{2\alpha}$  from the uterus of the guinea-pig. Donovan (1961) found that oxytocin treatment had no effect on cycle length when administered to guinea-pigs. This was further confirmed when oxytocin was shown to have no effect on prostaglandin production from either day 7 or 15 superfused guinea-pig uterus *in vitro* (Poyser and Brydon, 1983) or from cultured endometrial tissue (Riley and Poyser, 1987b). However, the latter study is in contradiction to a study by Leaver and Seawright (1982). A more recent study has demonstrated that oxytocin binding sites are present in the guinea-pig ovary and are regulated by steroid hormones so that oxytocin may play a role in follicular steroidogenesis, maturation or ovulation rather than in luteal function. Oxytocin binding sites were present in the guinea-pig uterus, but their numbers only varied very slightly during the oestrous cycle (Zhang, Dreifuss, Dubois-Dauphin and Tribollet, 1991).

### **1:10:1 Oxytocin and ovarian steroids**

Oxytocin was found to increase greatly  $\text{PGF}_{2\alpha}$  levels in the posterior vena cava of anoestrous sheep only if they had been previously oestradiol-primed. This suggested that the stimulation of uterine  $\text{PGF}_{2\alpha}$  release by oxytocin may depend

upon the presence of oestradiol (Sharma and Fitzpatrick, 1974). Oestradiol-priming was also shown to increase greatly uterine  $\text{PGF}_{2\alpha}$  release following oxytocin treatment in ovariectomized rats (Campos *et al.*, 1980) and ovariectomized rabbits (Small *et al.*, 1978). Previous workers had shown that the sensitivity of the rat uterus to oxytocin increased towards the end of the cycle (Chan, O'Connell and Pomeroy, 1963) and following oestrogen treatment (Follet and Bentley, 1964). Soloff and Swartz (1974) demonstrated that the rat uterus bound oxytocin with high affinity and that oestradiol treatment increased the number of oxytocin binding sites present in the ovariectomized rat uterus after only 6h (Soloff, 1975). It was then shown that the quantity of high affinity oxytocin receptors in sheep uterus increased after day 14 of the oestrous cycle, reaching a peak at oestrus. The endometrium was found to have a binding capacity twice that of the myometrium (Roberts *et al.*, 1976). This led to the proposition that oestradiol levels could regulate endometrial  $\text{PGF}_{2\alpha}$  release by controlling the availability of oxytocin receptors (McCracken, 1980). However, the sheep endometrium only became responsive to oxytocin after plasma progesterone levels had begun to decline so that progesterone appears to inhibit the rise in oxytocin receptors induced by oestrogen (Sheldrick and Flint, 1985; Roberts *et al.*, 1976). Therefore in the sheep, oestrogen acting on a progesterone-primed uterus appears to be the initial stimulus for increased endometrial  $\text{PGF}_{2\alpha}$  release and once progesterone levels have started to decrease the endometrium becomes responsive to oxytocin which may play an important role in the maintenance of uterine  $\text{PGF}_{2\alpha}$  output during luteolysis in the sheep. Several studies have proposed that the oxytocin-induced stimulation of ovine endometrial PG synthesis may be mediated through its ability to stimulate a rapid increase in the activity of PLC (Flint, Leat, Sheldrick and Stewart, 1986a; Silvia and Homanics, 1988).

#### **1:10:2 Oxytocin and phospholipase (PL) C**

Activation of PLC results in the hydrolysis of phosphoinositides, releasing diacylglycerol (DAG) and inositol phosphates ( $\text{IP}_3$ ) (Berridge, 1987). DAG can act as a second messenger through the activation of PKC which may phosphorylate, and therefore activate, enzymes involved in liberating free arachidonic acid from storage pools to serve as a substrate for PG synthesis. The involvement of PLC was confirmed when agonists that mimic the effects of DAG and  $\text{IP}_3$  enhanced  $\text{PGF}_{2\alpha}$  release from ovine (Silvia and Homanics, 1988; Silvia, Lee, Trammell, Hayes, Lowberger and Brockman, 1994) and bovine (Lafrance and Goff, 1990) endometrial tissue. Alternatively, it was proposed that arachidonic acid may be cleaved directly from DAG by DAG and monoacylglycerol lipases (Flint *et al.*, 1986a). However, recent work has demonstrated that it is difficult to establish a definitive association



between the level of PLC activity and the amount of  $\text{PGF}_{2\alpha}$  released *in vitro* in the ewe (Silvia *et al.*, 1994). Considerable  $\text{PGF}_{2\alpha}$  release can occur from ovine and bovine endometrial tissue with little or no detectable increase in PLC (Raw and Silvia, 1991; Mirando, Becker and Whiteaker, 1993; Silvia and Raw, 1993).

### **1:10:3 Oxytocin and phospholipase (PL) $\text{A}_2$**

$\text{AlF}_4^-$  is a potent stimulator of  $\text{PGF}_{2\alpha}$  release from ovine endometrial tissue (Silvia *et al.*, 1994) which appears to induce cellular responses by increasing the activity of G-proteins (Gilman, 1987). G-proteins can interact with several enzymes that could contribute to the regulation of endometrial  $\text{PGF}_{2\alpha}$  synthesis, such as adenylate cyclase, PLC and  $\text{PLA}_2$  (Gilman, 1987; Fain, Wallace and Wojcikiewicz, 1988). However, oxytocin did not induce an accumulation of cAMP in ovine endometrial tissue (Vallet and Bazer, 1989) and cAMP had no stimulatory effect on the release of  $\text{PGF}_{2\alpha}$  by bovine (Lafrance and Goff, 1990) or guinea-pig (Poyser, 1987a) endometrial tissue *in vitro*. Therefore, adenylate cyclase does not appear to be involved in the stimulation of  $\text{PGF}_{2\alpha}$  synthesis by oxytocin in the endometrium.

Previous work has demonstrated that the synthesis of PGs is associated with an increase in the activity of  $\text{PLA}_2$  in numerous cell types (Irvine, 1982). This increase in activity could be induced by an increase in PLC (and associated second messengers) or through direct activation from the ligand-bound receptor through a G-protein (Fain *et al.*, 1988). Studies on ovine endometrial tissue using melittin (a stimulator of  $\text{PLA}_2$ ) and aristolochic acid (an inhibitor of  $\text{PLA}_2$ ) have demonstrated that the stimulatory effect of oxytocin on endometrial  $\text{PGF}_{2\alpha}$  release is mediated through  $\text{PLA}_2$  (Lee and Silvia, 1994). However, the precise point at which  $\text{PLA}_2$  fits in the regulatory cascade triggered by oxytocin remains to be determined.

### **1:11 Pulsatile release of $\text{PGF}_{2\alpha}$**

The output of  $\text{PGF}_{2\alpha}$  from the uterus has been shown to occur in a pulsatile, not continuous, manner in the sheep (Thorburn, Cox, Currie, Restall and Schneider, 1972; McCracken *et al.*, 1972), cow (Nancarrow *et al.*, 1973), pig (Gleeson and Thorburn, 1973; Gleeson *et al.*, 1974) and goat (Homeida and Cooke, 1982). In the sheep at the time of luteolysis,  $\text{PGF}_{2\alpha}$  output was found to occur as a series of high amplitude, short duration pulses (Thorburn *et al.*, 1973; Barcikowski *et al.*, 1974; Baird *et al.*, 1976; Fairclough, Moore, McGowan, Peterson, Smith, Tervit and Watkins, 1980; Flint and Sheldrick, 1983; Zarco, Stabenfeldt, Quirke, Kindahl and Bradford, 1988a).

The minimal effective luteolytic dose of  $\text{PGF}_{2\alpha}$  when administered as a continuous infusion into the arterial supply of sheep ovary was found to be in the

range of 2  $\mu\text{g/h}$  (Goding, Baird, Cumming and McCracken, 1971). When  $\text{PGF}_{2\alpha}$  was infused as pulses into the autotransplanted sheep ovary (at a frequency calculated to mimic physiological exposure of the *in situ* ovary to  $\text{PGF}_{2\alpha}$  during naturally occurring luteolysis), it was found that 4 pulses of 1 h each (0.04 - 0.08  $\mu\text{g/ml}$ ) in a 19 h period caused permanent luteal regression in 25 % of animals. However, when 5 pulses were given in a total period of 25 h, permanent luteal regression occurred in all animals (Schramm, Bovaird, Glew, Schramm and McCracken, 1983). The average total effective dose of  $\text{PGF}_{2\alpha}$  required to induce luteal regression when administered as 5 x 1 h pulses was equal to 1/40th of the regarded effective dose when administered as a continuous infusion into the ovarian artery. These low concentrations of  $\text{PGF}_{2\alpha}$  were proposed to be comparable to quantitative estimates of  $\text{PGF}_{2\alpha}$  concentrations exchanged by the counter-current mechanisms in the utero-ovarian vein in the sheep (McCracken, Schramm and Okulicz, 1984b). A pulsatile infusion administering 1 pulse per day over 4 days failed to induce permanent regression indicating that a relatively short pulse frequency over a minimal period of around 24 h is the probable stimulus for physiological luteal regression in the sheep (Schramm *et al.*, 1983).

### **1:12 Control of pulsatile $\text{PGF}_{2\alpha}$ release**

The pulsatile release of  $\text{PGF}_{2\alpha}$  during luteal regression was originally thought to be linked to oxytocin secretion from the posterior pituitary (Roberts and McCracken, 1976; Fairclough *et al.*, 1980). Oxytocin itself is released in a pulsatile manner in the sheep (Mitchell, Kramer, Brennecke and Webb, 1982) and goat (Homeida and Cooke, 1983). However, several studies demonstrated that it was doubtful that an increase in oxytocin concentrations at the time of luteolysis stimulated uterine  $\text{PGF}_{2\alpha}$  release in cycling sheep since oxytocin and progesterone levels decreased simultaneously at the time of oestrus (Webb, Mitchell, Falconer and Robinson, 1981; Sheldrick and Flint, 1981; Schams, Lahlou-Kassi and Glatzel, 1982). This close association between oxytocin and progesterone levels led to the proposition that the ovary was possibly responsible for oxytocin secretion. Indeed, high oxytocin concentrations were then reported in both ovine and bovine corpora lutea (Wathes and Swann, 1982; Fields, Eldridge, Fuchs, Roberts and Fields, 1983) and a later study demonstrated that the large luteal cells were responsible for oxytocin synthesis and secretion (Rodgers, O'Shea, Findlay, Flint and Sheldrick, 1983b). Cloprostenol (a  $\text{PGF}_{2\alpha}$  analogue) administration stimulated oxytocin secretion into the ovarian vein in the sheep. Oxytocin concentrations decreased at oestrus, but frequent sampling revealed that the majority of pulses of uterine  $\text{PGF}_{2\alpha}$  were

accompanied by rises in peripheral plasma oxytocin concentrations (Flint and Sheldrick, 1982a, b) which suggested that  $\text{PGF}_{2\alpha}$  and oxytocin secretion may be linked by a positive feedback mechanism (Flint and Sheldrick, 1983). This was further supported by the observation that following hysterectomy in the sheep, oxytocin secretion decreased from the corpora lutea indicating that oxytocin requires the presence of endogenous uterine  $\text{PGF}_{2\alpha}$  for its secretion (Sheldrick and Flint, 1983). However, although cloprostenol administration did not increase oxytocin concentrations in ovarian and jugular venous plasma in hysterectomized sheep, luteal regression was not delayed so that oxytocin secreted from the corpora lutea does not appear to be involved in ovarian events mediating  $\text{PGF}_{2\alpha}$ -induced luteolysis in the sheep.

Similar findings have been reported in the goat. Decreasing peripheral levels of oxytocin from day 12 are characterised by frequent pulses of oxytocin (Homeida and Cooke, 1983). Indomethacin treatment on days 11 and 16 of the cycle delayed luteolysis, prevented the decrease in oxytocin concentrations and suppressed the pulsatile appearance of both oxytocin and  $\text{PGF}_{2\alpha}$  metabolite in the peripheral circulation (Cooke and Homeida, 1984). No effect on peripheral oxytocin or  $\text{PGF}_{2\alpha}$  metabolite concentrations was observed in ovariectomized goats following  $\text{PGF}_{2\alpha}$  administration (Homeida and Cooke, 1985), suggesting that uterine  $\text{PGF}_{2\alpha}$  may induce the pulsatile release of ovarian oxytocin during luteolysis.

In the sheep, pulses of  $\text{PGF}_{2\alpha}$  occur concurrently with pulses of oxytocin (or its associated neurophysin) during luteal regression (Fairclough *et al.*, 1980; Webb *et al.*, 1981; Flint and Sheldrick, 1983). Further work revealed that the majority of this oxytocin is secreted by the corpus luteum (Hooper, Watkins and Thorburn, 1986; Moore, Choy, Elliot and Watkins, 1986). Uterine  $\text{PGF}_{2\alpha}$  and luteal oxytocin appear to comprise a positive feedback loop (Flint and Sheldrick, 1983). Indeed, oxytocin can stimulate uterine secretion of  $\text{PGF}_{2\alpha}$  (Sharma and Fitzpatrick, 1974; Roberts *et al.*, 1975; Roberts and McCracken, 1976) and  $\text{PGF}_{2\alpha}$  can stimulate secretion of oxytocin from the corpus luteum (Flint and Sheldrick, 1982b; Watkins and Moore, 1987; Heap, Fleet, Davies, Goode, Hamon, Walters and Flint, 1989; Lamsa *et al.*, 1989). Flint *et al.* (1986) proposed that the high concentrations of both  $\text{PGF}_{2\alpha}$  and oxytocin were achieved through activation of this positive feedback loop. At the time of luteolysis, concentrations of  $\text{PGF}_{2\alpha}$  increase in the utero-ovarian venous effluent before any detectable increase in oxytocin (Moore *et al.*, 1986) implying that the activation of the feedback loop begins on the uterine side of the loop. The actual stimulus that initiates  $\text{PGF}_{2\alpha}$  secretion is still unclear. In sheep, pulses of  $\text{PGF}_{2\alpha}$  are frequently associated with increases in both neurohypophyseal and luteal secretion of

oxytocin (Hooper *et al.*, 1986). McCracken, Smith, Lamsa and Robinson (1991) suggested that neurohypophyseal oxytocin, released in a pulsatile fashion, could serve as a pulse generator signal initiating uterine secretion of  $\text{PGF}_{2\alpha}$ .

Ovarian steroid hormones may also contribute to the regulation of the secretion of pulsatile  $\text{PGF}_{2\alpha}$ . Pulsatile secretion of uterine  $\text{PGF}_{2\alpha}$  is greatly reduced in the absence of ovarian steroids (Silvia and Raw, 1993b). Changes in the uterine responsiveness to oxytocin appear to be regulated by oestradiol and progesterone (Sharma and Fitzpatrick, 1974; McCracken, 1980; Homanics and Silvia, 1988; Vallet, Lamming and Batten, 1990a). Oestradiol and progesterone may also influence the "pulse generator" since infusion of oestradiol into ovariectomized ewes resulted in the secretion of a pulsatile pattern of neurohypophyseal oxytocin similar to that observed in intact ewes during the follicular phase (McCracken, Schramm, Manning and Robinson, 1984a; McCracken *et al.*, 1984b). A more recent study has demonstrated that progesterone plays an important role in regulating the number and magnitude of secreted pulses of  $\text{PGF}_{2\alpha}$  from the ovine uterus (Silvia and Raw, 1993b). The administration of progesterone to ovariectomized ewes completely restored the frequency, and partially restored the magnitude, of uterine  $\text{PGF}_{2\alpha}$  pulses, suggesting that ovarian secretory products other than progesterone (such as luteal oxytocin) are not required to initiate pulsatile secretion of  $\text{PGF}_{2\alpha}$ , but may be necessary to achieve full pulse magnitude. A role of oestradiol in regulating pulsatile secretion of  $\text{PGF}_{2\alpha}$  was not evident (Silvia and Raw, 1993b).

The mechanism by which the eventual termination of both oxytocin and  $\text{PGF}_{2\alpha}$  secretion is achieved was proposed to be due to the down-regulation of oxytocin receptors by oxytocin (Flint and Sheldrick, 1985), since a continuous intravenous infusion of oxytocin between days 13 and 21 after oestrus in the sheep delays the return to oestrus and prevents the increase in uterine oxytocin receptors that normally precedes oestrus. However, in a later study, Sheldrick and Flint (1986) treated steroid-primed ovariectomized sheep with oxytocin at 1, 2, 4 or 6 h intervals. All initial doses elicited large increases in circulating  $\text{PGF}_{2\alpha}$  metabolite. The subsequent doses failed to result in such high increases, although the response was higher when the time period between doses was greater. This suggested that a period of uterine refractoriness existed following administration of oxytocin in steroid-primed ovariectomized sheep. Since uterine oxytocin receptor numbers remained unchanged after 2 h of oxytocin treatment, this indicated that uterine refractoriness to oxytocin was not due to down-regulation of oxytocin receptors (Sheldrick and Flint, 1986). The timing of the period of uterine refractoriness following oxytocin treatment was consistent with the frequency of uterine  $\text{PGF}_{2\alpha}$  pulses secreted at luteolysis, which



have been reported to occur at intervals of greater than 6 h (Sheldrick and Flint, 1986). This was also consistent with the frequency with which  $\text{PGF}_{2\alpha}$  must be administered to obtain enhanced luteolytic potency (Schramm *et al.*, 1983).

The outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the guinea-pig uterus superfused *in vitro* were shown to increase following treatment with phospholipase (PL)  $\text{A}_2$ , the calcium ionophore A23187 and arachidonic acid (Poyser and Brydon, 1983; Poyser, 1987). When the same treatment was applied 1 h after termination of the first treatment,  $\text{PLA}_2$  failed to increase uterine prostaglandin output, A23187 caused a smaller increase, but arachidonic acid treatment produced the same large increase in uterine PG output. Therefore, the uterus demonstrated complete refractoriness to  $\text{PLA}_2$ , partial refractoriness to A23187 and no refractoriness to arachidonic acid with respect to the stimulation of uterine PG output. However, this refractoriness only lasts for 3-5 h (Poyser, 1991). Since there is no failure with time in the mechanisms that convert exogenous arachidonic acid into PGs, the refractoriness of uterine PG production seems to occur at the level of endogenous arachidonic acid release. This would indicate that there are one or several pools of bound arachidonic acid which are readily releasable but which take 3-5 h to refill, and this may explain, in part, the pulsatile nature of uterine  $\text{PGF}_{2\alpha}$  release.

### 1:13 Hormonal control of uterine receptors

Progesterone treatment has been shown to inhibit the replenishment of uterine oestrogen receptors in the immature, oestradiol-treated rat (Hsueh, Peck and Clark, 1976), human (Tseng and Gurpide, 1975), ovariectomized oestradiol-treated monkey (Brenner, Resko and West, 1974), and rat (West, Verhage and Brenner, 1976). In ovine endometrium, progesterone prevented the increase in oxytocin receptors normally stimulated by oestradiol (Koligian and Stormshak, 1977a), and when circulating progesterone concentrations were maximal, during the mid-luteal phase, oestrogen receptor concentrations were found to be at their lowest (Koligian and Stormshak, 1977b). Progesterone has also been shown to rapidly and selectively down-regulate oestrogen receptors in the hamster (Evans and Leavitt, 1980) and rat uterus (Okulicz, Evans and Leavitt, 1981). Ovariectomized guinea-pigs treated with oestradiol exhibited an increase in the concentration of uterine progesterone receptors, this increase being prevented by pretreatment with protein synthesis or RNA synthesis inhibitors. However, when progesterone was administered following pretreatment with oestradiol, a marked decrease in progesterone receptor concentration resulted. This was unaffected by protein synthesis inhibitors, indicating enhanced receptor inactivation as a possible mechanism, such as induction of

translocation of receptors to the cytosol (Vu Hai, Logeat, Warembourg and Milgrom, 1977). Therefore, the down-regulation of progesterone receptors could explain the loss of the inhibitory action of progesterone on oestradiol seen towards the end of the oestrous cycle in the sheep uterus (McCracken *et al.*, 1984a).

There is considerable evidence that the concentration of uterine oxytocin receptors is regulated principally by the circulating steroid hormones (McCracken *et al.*, 1984b; Sheldrick and Flint, 1985), with oestradiol tending to induce receptor formation and progesterone having an initial inhibitory effect which switches to an enhancement after 10-12 days (Vallet *et al.*, 1990; Zhang, Weston and Hixon, 1992). Uterine oxytocin receptors have been shown to be present in ovariectomized ewes (Vallet *et al.*, 1990; Zhang *et al.*, 1992). However, these receptors are functionally inactive as oxytocin administration is unable to stimulate  $\text{PGF}_{2\alpha}$  release, presumably because other essential elements in the signalling pathway are steroid-dependent (Flint *et al.*, 1986; Hixon and Flint, 1987). Initial treatment of ovariectomized ewes with progesterone down-regulates the oxytocin receptor, but this effect is lost with time, so that receptors reappear after about 12 days of continuous progesterone treatment (Homanics and Silvia, 1988; Vallet *et al.*, 1990). It was proposed that maximal responses in terms of both oxytocin receptor concentration and  $\text{PGF}_{2\alpha}$  release in the ewe can be achieved by a period of progesterone treatment, followed by oestradiol administration during progesterone withdrawal (Vallet *et al.*, 1990; Zhang *et al.*, 1992). This hypothesis was supported by a previous study by Homanics and Silvia (1988) which demonstrated that oxytocin administration on days 5 or 10 of steroid treatment did not result in any change in  $\text{PGF}_{2\alpha}$  metabolite (PGFM) concentration in the jugular venous blood of ovariectomized ewes treated with either oestradiol, progesterone or both steroids. When oxytocin was administered on day 15 of progesterone treatment, circulating PGFM levels did increase. However, a greater increase in PGFM levels was observed in animals that had been treated with both progesterone and oestradiol. A later study in 1990 by Ayad, McGogg and Wathes also provided evidence that progesterone and oestradiol treatment together resulted in maximal oxytocin binding. They found that oxytocin binding to oviductal membranes in prepubertal, anoestrous and pregnant ewes was very low. However, in anoestrous ewes treated with both steroids, binding was similar to that measured in normal ewes at oestrus. Therefore in the ewe, it appears that the hormonal stimuli that maximally induce uterine  $\text{PGF}_{2\alpha}$  release towards the end of the cycle, also induce maximum oxytocin binding to reproductive tissues thereby further stimulating uterine  $\text{PGF}_{2\alpha}$  release and maintaining the process of luteolysis. Progesterone appears to have the dominant role in the control of luteolysis in the ewe since long-term progesterone

treatment alone was found to be sufficient to induce both endometrial oxytocin receptors and uterine  $\text{PGF}_{2\alpha}$  release in response to oxytocin administration. Oestrogen may simply mediate the time course of  $\text{PGF}_{2\alpha}$  release in response to oxytocin, maintaining luteolysis as progesterone levels decrease in response to  $\text{PGF}_{2\alpha}$  (Vallet et al., 1990).

A recent study has also suggested that local paracrine factors, as well as circulating steroid hormone concentrations, are likely to be involved in the regulation of oxytocin receptors during the oestrous cycle of the ewe (Wathes and Hamon, 1993). Clearly, the mechanisms by which oxytocin receptor number and function are regulated, have yet to be fully elucidated.

To summarise, the main evidence supporting the theory that  $\text{PGF}_{2\alpha}$  is the uterine luteolytic hormone in many species:-

- i. Administration of  $\text{PGF}_{2\alpha}$  mimics the action of the "uterine luteolytic factor" resulting in luteolysis.
- ii.  $\text{PGF}_{2\alpha}$  has been detected in the uterine vein prior to and during luteolysis.
- iii. The insertion of intrauterine devices or beads which induce premature luteolysis stimulate an early release of  $\text{PGF}_{2\alpha}$  from the uterus.
- iv. Indomethacin administration which inhibits  $\text{PGF}_{2\alpha}$  synthesis prolongs luteal function.
- v. Active or passive immunization against  $\text{PGF}_{2\alpha}$  results in luteal maintenance.
- vi.  $\text{PGF}_{2\alpha}$  has been shown to have a luteolytic effect on luteal cells.
- vii.  $\text{PGF}_{2\alpha}$  has been shown to be transferred from the uterus to the ovary.

#### **1:14 $\text{PGF}_{2\alpha}$ in the early pregnant animal**

Implantation in the guinea-pig is completed during a period of 6-8 hours on the sixth day following fertilization (Blandau, 1949). The terms "superficial" and "interstitial" describe different implantation mechanisms, and separate those in which the blastocyst remains in the uterine lumen and those in which it penetrates the luminal epithelium and embeds itself in the uterine wall. The guinea-pig blastocyst was first classed as one demonstrating a true type of interstitial implantation by Spee in 1883.

Both man and the guinea-pig have a "discoid haemochorial placenta" in which maternal blood circulates through trophoblastic channels. In the guinea-pig, this discoid haemochorial placenta (chorio-allantoic placenta) rests on a base called the subplacenta (Egund and Carter, 1974), or yolk sac or vitelline placenta (Schroder, Schoch, Elwers and Leichtweiss, 1991). During implantation in guinea-pigs (and rat, rabbit, hamster and mouse) the yolk sac membrane gains contact with the endometrial



epithelium forming the yolk sac placenta (Schroder *et al.*, 1991). In guinea-pigs, the resorptive (luminal) microvillous side of the yolk sac epithelium forms extensive interdigitations with the apical microvilli of the endometrial epithelium (Kaufmann and Davidoff, 1977) through a process termed "yolk sac inversion". The cells that constitute the subplacenta are derived from fetal endodermal cells (Schroder *et al.*, 1991).

Ovarian progesterone is necessary for the maintenance of pregnancy, so it is essential that the regression of the corpus luteum is prevented after a successful mating. Consequently, in those animals that secrete a uterine luteolytic hormone, the luteolytic influence of the uterus must be abolished in some way following conception. Pregnant guinea-pigs, ovariectomized on day 21 of gestation, aborted unless treated with progesterone. However, if ovariectomy was carried out on day 30 of gestation, none of the animals aborted (Heap and Deanesly, 1966; Csapo, Puri and Tarro, 1981). Therefore, in the guinea-pig, ovarian progesterone is necessary to maintain pregnancy until about day 25, in a 67-day gestation. The corpora lutea of the guinea-pig are actually maintained until days 58-67, but are not essential for the maintenance of pregnancy beyond day 25 (Bland and Donovan, 1969b). Around day 25, the "luteo-placental shift" occurs when the placenta takes over progesterone production. However, the corpora lutea remain fully functional until about day 40 after which progesterone secretion gradually declines (Heap and Deanesly, 1966). A similar situation exists in the sheep. In some other species, such as the cow, pig and goat, ovarian progesterone is essential throughout gestation. In pregnant guinea-pigs, the usual increase in  $\text{PGF}_{2\alpha}$  output from the non-pregnant uterus after day 11 is prevented by the presence of the conceptuses (Blatchley, Maule Walker and Poyser, 1975a, b; Antonini *et al.*, 1976). Conceptuses from pregnant guinea-pigs grafted on to the spleen of non-pregnant animals maintained corpora lutea in the recipient guinea-pigs beyond their normal life-span (Bland and Donovan, 1969b). The low concentrations of  $\text{PGF}_{2\alpha}$  present in the utero-ovarian vein of pregnant guinea-pigs were not due to increased metabolism of prostaglandins, lack of substrate or the redirection of synthesis towards  $\text{PGE}_2$  (Maule Walker and Poyser, 1978). Therefore, it was suggested that the conceptus secretes an anti-luteolytic factor which may suppress uterine  $\text{PGF}_{2\alpha}$  output by inhibiting the stimulatory effect of oestradiol on endometrial  $\text{PGF}_{2\alpha}$  synthesis and release (Poyser, 1984a). This anti-luteolytic factor was previously shown to act both locally and systemically since both uterine horns from day 15 unilaterally pregnant guinea-pigs produced significantly less  $\text{PGF}_{2\alpha}$  *in vitro* than horns from day 15 non-pregnant animals. However the local anti-luteolytic action is stronger than the systemic action since the non-pregnant horns of the

unilateral pregnancies produced significantly more  $\text{PGF}_{2\alpha}$  than the pregnant horns (Maule Walker and Poyser, 1974).

During early pregnancy, luteal maintenance is associated with the production of antiluteolytic proteins by the embryonic trophoblast between days 13-21 in the ewe (Godkin, Bazer, Moffatt, Sessions and Roberts, 1982), cow (Bartol, Roberts, Bazer, Lewis, Godkin and Thatcher, 1984) and goat (Gnatek, Smith, Duby and Godkin, 1989). In the sheep, the embryo secretes a protein called ovine trophoblast protein-1 (oTP-1) which has been shown to inhibit oestradiol- and oxytocin-induced stimulation of endometrial  $\text{PGF}_{2\alpha}$  synthesis *in vivo* (Vallet, Bazer, Fliss and Thatcher, 1988). oTP-1 has a molecular weight of 20 kDa and has approximately 70 % homology with the  $\alpha$ -interferons (Imakawa, Anthony, Kayemi, Marotti, Polites and Roberts, 1987; Stewart, McCann, Barker, Lee, Lamming, Flint, 1987; Charpigny, Reinaud, Huet, Guillomot, Charlier, Pernollet and Martal, 1988).  $\alpha$ -Interferon also strongly inhibits  $\text{PGF}_{2\alpha}$  synthesis by sheep endometrial cells (Salamonsen, Manikhot, Healy and Findlay, 1989; Salamonsen, Stuchberg, O'Grady, Godkin and Findlay, 1988). During the oestrous cycle and early pregnancy oTP-1 binding sites are present in the sheep endometrium (Knickerbocker and Niswender, 1989) and interferon- $\alpha$  will also bind to these sites (Stewart *et al.*, 1987). A similar protein, called bovine trophoblast protein (bTP-1) (Mr 21 kDa), is secreted by bovine embryos. bTP-1 shows considerable homology with oTP-1 and also inhibits endometrial  $\text{PGF}_{2\alpha}$  synthesis (Gross, Plante, Thatcher, Hansen, Helmer and Putney, 1988c; Lifsey, Braumbach and Godkin, 1989). Intra-uterine and systemic administration of recombinant bovine interferon- $\alpha_1$  at the time of maternal recognition of pregnancy to cyclic ewes can also act as a antiluteolysin, apparently acting in a similar way to oTP-1 (Parkinson, Lamming, Flint and Jenner, 1992). In cattle, bTP-1 appears to inhibit both basal, and oxytocin- and oestradiol-stimulated uterine  $\text{PGF}_{2\alpha}$  secretion (Thatcher, Danet-Desnoyers and Wetzels, 1992), whereas in sheep oTP-1 may inhibit the pulsatile secretion of  $\text{PGF}_{2\alpha}$  although this is accompanied by an increase in basal  $\text{PGF}_{2\alpha}$  secretion (Inskeep and Murdoch, 1980). In order to distinguish trophoblast protein-1 from other types of interferon, these trophoblast proteins have been recently renamed interferon- $\tau$  (see Poyser, 1995). Another study has proposed that embryo-derived platelet-activating factor may play a role in suppressing uterine  $\text{PGF}_{2\alpha}$  secretion in the sheep (Battye, O'Neill and Evans, 1991).

The conceptus of the pig has also been demonstrated to secrete an interferon-like protein during early pregnancy (Cross and Roberts, 1989). However, proteins secreted by the conceptus do not inhibit uterine  $\text{PGF}_{2\alpha}$  release in the pig *in vivo* (Harney and Bazer, 1989). The secretion of oestradiol by the pig embryo appears to

induce an alteration in  $\text{PGF}_{2\alpha}$  secretion, whereby  $\text{PGF}_{2\alpha}$  is secreted into the uterine lumen (where it is sequestered) rather than into the uterine venous drainage (Bazer and Thatcher, 1977; Bazer, Mirando, Ott, Harney, Dubois, Schalue, Pontzer, Hostetler, Johnson and Ogle, 1992). Therefore, the corpora lutea remain protected from the luteolytic influence of  $\text{PGF}_{2\alpha}$ . Oestradiol-induced calcium cycling and synergistic effects of prolactin may be involved in this redirection of  $\text{PGF}_{2\alpha}$  secretion (Bazer *et al.*, 1992).

Administration of  $\text{PGF}_{2\alpha}$  induced luteolysis in nonpregnant but not in pregnant sheep (Nancarrow, Evison and Connell, 1982; Silvia and Niswender, 1986) even though the number of  $\text{PGF}_{2\alpha}$  receptors was similar in both at the time of maternal recognition of pregnancy (Wiepz, Wiltbank, Nett, Niswender and Sawyer, 1992). In addition, the responsiveness of large luteal cells to  $\text{PGF}_{2\alpha}$  was not different between pregnant and nonpregnant animals (Wiltbank, Wiepz, Knickerbocker, Braden, Sawyer, Mayan and Niswender, 1992a). Therefore, inhibition of the action of  $\text{PGF}_{2\alpha}$  may occur at the level of the luteal cell. It has been proposed that the pregnant uterus secretes a factor, possibly  $\text{PGE}_2$ , that reduces the luteolytic effects of  $\text{PGF}_{2\alpha}$ .  $\text{PGE}_2$  treatment lengthened the lifespan of the corpus luteum in sheep (Inskeep, Smutny, Butcher and Pexton, 1975), cattle (Gimenez and Henricks, 1983), primates (Zelinski-Wooten and Stouffer, 1990) and stimulated progesterone production by ovine large luteal cells (Fitz, Hoyer and Niswender, 1984).

Other factors that may be involved during maternal recognition of pregnancy are luteal protective proteins secreted by the early ovine embryo (Wiltbank, Wiepz, Knickerbocker, Belfiore and Niswender, 1992b). These conceptus proteins did not directly stimulate progesterone production, but appeared to block the action of  $\text{PGF}_{2\alpha}$  on large luteal cells. Since there was no change in binding of  $\text{PGF}_{2\alpha}$  to its receptor at the same doses of conceptus protein that completely inhibited the antisteroidogenic action of  $\text{PGF}_{2\alpha}$ , secreted conceptus proteins appear to act after activation of free calcium / PKC intracellular effector pathways and may have general cellular protective actions. oTP-1 failed to exhibit such anti- $\text{PGF}_{2\alpha}$  activity, but conceptus proteins devoid of oTP-1 did prevent the antisteroidogenic effects of  $\text{PGF}_{2\alpha}$ . Therefore, secreted conceptus proteins may be important in maintaining the corpus luteum during early pregnancy in the sheep (Wiltbank *et al.*, 1992b).

In the guinea-pig, interferon- $\alpha$  does not inhibit  $\text{PGF}_{2\alpha}$  or  $\text{PGE}_2$  production by day 15, non-pregnant endometrium in culture (Leckie and Poyser, 1990a). Proteins related to interferon- $\alpha$  appear to act as anti-luteolytic factors during early pregnancy by inhibiting endometrial  $\text{PGF}_{2\alpha}$  synthesis in some species (sheep and cow) but not in others such as the pig and guinea-pig.

In the human and other primates the luteotrophic stimulus is provided by a placental protein hormone chorionic gonadotrophin (CG) (Hobson, 1971). In the guinea-pig, hypophysectomy 3-6 days after mating (Heap, Perry and Rowlands, 1967) or ovariectomy 3-4 weeks post coitum (Herrick, 1928) does not result in abortion. Therefore, corpora lutea of pregnancy and the production of progesterone must be maintained by an extra-pituitary source of luteotrophin. Bland and Donovan (1969) demonstrated that the transfer of conceptuses to, and their subsequent removal from, the uterus of guinea-pigs resulted in first the prevention and secondly the recurrence of oestrus. They concluded that a placental hormone, whose nature was unknown, was responsible for continued luteal function and the absence of oestrus during pregnancy in the guinea-pig. In an earlier study by Davies, Dempsey and Amoroso (1961) investigating the development of the guinea-pig subplacenta throughout pregnancy, it was concluded that this tissue may be the source of a gonadotrophin. A later study successfully extracted a gonadotrophic substance from placentae and subplacentae of days 10-63 pregnant guinea-pigs (Humphreys, Hobson and Wide, 1982). A subsequent study demonstrated that the guinea-pig placenta produces a CG, which on purification has physiochemical, biological and immunological properties similar to those of human CG (hCG) (Bambra, Lynch, Foxcroft, Robinson and Amoroso, 1984). The concentration of CG in the guinea-pig placenta plus subplacenta is approximately 1/100th of that found in the human placenta, however, if the CG would reach the ovary via a local veno-arterial pathway such a low concentration may be sufficient to maintain the corpus luteum (Humphreys *et al.*, 1982).

Biologically active gonadotrophins with gonadotrophin profiles similar to hCG and its subunits have been shown to be present in the placentae of rat, mouse and hamster (Wide and Hobson, 1978), and implanting mouse blastocysts synthesize and release a glycoprotein allied to CG (Fishel and Surani, 1980). The administration of hCG reduced the luteolytic effect of PGF<sub>2α</sub> in the ewe (Bolt, 1979). The luteotrophic function of CG has been well described for several primate species, including man (see Hearn, Webley and Gidley-Baird, 1991). Passive immunization against hCG between days 20 and 25 after ovulation of 10 pregnant marmoset monkeys resulted in all pregnancies being terminated within 2-6 days. When marmoset embryos were cultured individually with added anti-hCGβ antiserum, attachment and trophoblastic outgrowth was prevented. These effects were not observed in embryos incubated with nonimmune IgG or in normal marmoset culture. These studies suggest that the neutralization of CG during embryo attachment *in vivo* and *in vitro* prevents implantation and terminates early pregnancy in the marmoset monkey (see Hearn *et al.*, 1991). Also, the direct perfusion of hCG into the corpora lutea of marmoset



monkeys produced an immediate stimulus resulting in a significant increase in progesterone production within 10-20 min (see Hearn *et al.*, 1991) strengthening the evidence that CG has luteotrophic action. The first external secretion of CG by the human embryo is most probably in the late blastocyst stages (Fishel, Edwards and Evans, 1984).

The identity of the anti-luteolytic factor in the guinea-pig remains unconfirmed as yet. A 38.2 kDa protein secreted by the day 15 conceptus which inhibits endometrial  $\text{PGF}_{2\alpha}$  synthesis has been isolated (Leckie and Poyser, 1992). There is a strong similarity between hCG and CG extracted from the guinea-pig placenta, which therefore may have a luteotrophic function similar to that of hCG (Humphreys *et al.*, 1982). Whether the 38.2 kDa protein previously isolated from the guinea-pig conceptus is secreted particularly by the placenta, and whether it is related to hCG (37.6 kDa) is as yet unknown, and requires further study.

## **The Control of Prostaglandin Synthesis**

### **1:15 Mechanism by which ovarian hormones control $\text{PGF}_{2\alpha}$ synthesis**

The concentrations of  $\text{PGF}_{2\alpha}$  present in uterine tissues are very low and definitely inadequate to account for the large increase in  $\text{PGF}_{2\alpha}$  released from the uterus towards the end of the cycle in the guinea-pig (Poyser, 1972) and sheep (Wilson, Cenedella, Butcher and Inskeep, 1972). Since PGs are not stored in the uterus, they must be immediately synthesized prior to their release. The mechanisms by which the ovarian steroids control this synthesis are discussed in this section.

#### **1:15:1 Steroid binding and protein synthesis**

In order to elicit a response, steroids must initially bind to their appropriate receptor. Steroid receptors were originally thought to exist as soluble constituents of the cytosol. However, studies with fluorescent antibody labelling (King and Greene, 1984) demonstrated that the receptor protein is bound to the nuclear chromatin. Steroid molecules cross the cell membrane readily since they are lipid soluble and bind to the receptor. The steroid-receptor complex is believed to bind to both the DNA and chromatin protein, the latter binding increasing the affinity of the interaction with the DNA. The receptor determines the specificity for a particular DNA sequence and thus regulates the formation of particular messenger RNAs, thereby directing the synthesis of specific proteins. A subsequent increase in general protein levels and an accumulation of phospholipids are observed following steroid administration (Mueller, Gorski and Aizawa, 1961). Intrauterine administration of actinomycin D (an inhibitor

of DNA-dependent RNA synthesis) on day 11 of the oestrous cycle prevented luteal regression in the sheep (French and Casida, 1973). This led to the suggestion that, towards the end of the cycle, oestradiol acted on a progesterone-primed uterus to stimulate DNA-dependent RNA synthesis resulting in a rise in the enzymes involved in PGF<sub>2α</sub> synthesis. Indeed, in the guinea-pig actinomycin D treatment prevented the normal rise in "uterine prostaglandin synthase enzymes" observed just prior to the time of luteal regression (Poyser, 1979). Later studies revealed that progesterone concentrations remained high during lengthened cycles and that PGF<sub>2α</sub> production by uterine homogenates *in vitro* on day 15 was reduced by 80-85 % in guinea-pigs which had received intrauterine actinomycin D on day 10 compared to control animals. None of the effects could be reversed by oestradiol treatment (Poyser and Riley, 1987). Actinomycin D, cycloheximide (which inhibits the elongation step of protein transcription) and puromycin (which releases nascent polypeptide chains prior to their complete synthesis) all reduced PGF<sub>2α</sub> output from both day 7 and 15 guinea-pig endometrium cultured for 24 h (Riley and Poyser, 1989). However, this effect was not observed when the inhibitors were superfused *in vitro* over the tissues for a 20 min period, indicating that these compounds do not have a rapid inhibitory effect on endometrial PG synthesis. Intrauterine administration of actinomycin D on day 10 reduced the synthesis of both secreted and cellular proteins by day 15 cultured endometrium suggesting that the increased endometrial synthesis of PGs, particularly PGF<sub>2α</sub> towards the end of the cycle, appears to be dependent upon increased endometrial protein synthesis.

#### **1:15:2 Influence of ovarian steroids on PGH synthase activity**

PGH synthase exhibits self-catalysed breakdown so that as PGs are formed, the enzyme catalysing the reaction is inactivated (Lands, LeTellier, Rome and Vanderhoek, 1972; Lands, 1979). Thus, the total amount of PGs synthesized by endometrial homogenates is indicative of the concentration of PGH synthase present, especially as the metabolism of PGs in the absence of exogenous NAD<sup>+</sup> by the guinea-pig uterus is negligible (< 5%) (Poyser, 1979). Increases in the amounts of PGF<sub>2α</sub> synthesized by homogenates of guinea-pig uterus towards the end of the cycle (Poyser, 1972) are paralleled by increases in PGF<sub>2α</sub> concentrations in the utero-ovarian vein (Blatchley *et al.*, 1972). This led to the proposition that increasing PG levels formed by the uterus may reflect an increase in the amount of PGH synthase present in the uterus (Poyser, 1973). Indeed, estimation of the amounts of [<sup>3</sup>H]-arachidonic acid converted into PGs by guinea-pig uterine microsomes confirmed that an increase in PG synthesizing activity did occur towards the end of the cycle (Wlodawer, Kindahl and Hamberg, 1976).

Various studies demonstrated that oestradiol treatment influenced the ratio of PG products synthesized by the uterus. Ovariectomy in the rat induced a 10-fold and 3-fold increase in uterine PGE and PGF release respectively. However, oestradiol treatment caused a decrease in PGE levels paralleled by an increase in PGF levels (Ham, Cirillo, Zanetti and Kuehl, 1975). Oestradiol was found to stimulate an increase in endometrial PGH synthase in the guinea-pig (Naylor and Poyser, 1975; Wlodawer *et al.*, 1976; Thaler-Dao, Ramonatxo, Saintot, Chaintreuil and Crastes de Paulet, 1982) and sheep (Raw, Curry and Silvia, 1988) uterus. Oestradiol treatment of ovariectomized guinea-pigs stimulated an increase in endometrial PG synthesizing capacity and increased the PGF<sub>2α</sub> to PGE<sub>2</sub> ratio whereas progesterone treatment had no effect on endometrial PG synthesizing capacity when administered alone. However, when both steroids were administered progesterone attenuated the stimulatory action of oestradiol in endometrial PG synthesizing capacity (Poyser, 1983a). A preferential stimulation of PGF<sub>2α</sub> synthesizing capacity in guinea-pig endometrium has been shown to occur after day 11 of the cycle (Poyser, 1983a), coupled with an increase in the amount of oestradiol released from the guinea-pig ovary after day 11 up to oestrus (Joshi *et al.*, 1973). This increase in ovarian oestradiol output would satisfactorily provide the stimulus for the increase in endometrial PGH synthase concentrations which occurs during the same period. However, for maximal PGF<sub>2α</sub> release to occur from the guinea-pig uterus superfused *in vitro*, a period of progesterone-priming must precede oestradiol treatment (Poyser, 1983b). The enzymatic capacity of the guinea-pig uterus to synthesize PGF<sub>2α</sub> appears to be stimulated by oestradiol and inhibited by progesterone, whereas the maximal synthesis and release of PGF<sub>2α</sub> seems to be dependent upon oestradiol acting on a progesterone-primed uterus.

Progesterone treatment increased the concentration of PGH synthase in uterine epithelial cells of ovariectomized sheep (Raw *et al.*, 1988) and rats (Ohta, 1985). Treatment of intact ewes with progesterone early in the oestrous cycle was also shown to induce a premature increase in the concentration of mRNA coding for PGH synthase in uterine tissue as well as premature luteolysis (Eggleston, Wilken, VanKirk, Slaughter and Murdoch, 1990). Therefore, progesterone appears to stimulate the synthesis of PGH synthase and this may explain the period of "progesterone-priming" required for maximal prostaglandin output.

However, changes in endometrial PGH synthase levels do not appear to be the only factor controlling endometrial PGF<sub>2α</sub> release at the time of luteolysis. PGF<sub>2α</sub> output from superfused guinea-pig uterus *in vitro*, increases 21.9-fold between days 7 and 15 of the cycle (Poyser and Brydon, 1983) whereas there is only a 2- to 3-fold



variation in the activity of PG synthesizing enzymes during the cycle (Poyser, 1972; Wlodawer *et al.*, 1976; Poyser, 1983a). It would therefore seem likely that an increase in the supply of free precursor arachidonic acid is a more probable stimulus for the increase in PGF<sub>2α</sub> synthesis by the endometrium at the time of luteolysis rather than an increase in PGH synthase activity.

### **1:15:3 Influence of ovarian steroids on phospholipase A<sub>2</sub> activity**

In the guinea-pig, free arachidonic acid available for PG synthesis only makes up 0.1 % of the total arachidonic acid present in the uterus (Leaver and Poyser, 1981). However, arachidonic acid esterified into membrane phospholipids is found in much greater quantities (1 mg/g uterine tissue). Progesterone has been shown to induce an accumulation of lipid droplets in ovine uterine epithelial cells (Brinsfield and Hawk, 1973) and an accumulation of phospholipids and triglycerides in rat endometrial tissue (Boshier and Holloway, 1973; Boshier, Holloway and Millener, 1981; Manimekalai, Umapathy and Govindarajulu, 1979) all being potential precursor pools for PGF<sub>2α</sub> synthesis. In the rat uterus, oestradiol treatment increased the rate of arachidonic acid turnover in both phospholipid and triglyceride storage pools (Toth and Hertelendy, 1986) and reduced the cytoplasmic area occupied by lipid droplets (Boshier and Holloway, 1973; Williams and Rogers, 1980). Therefore, the steroid hormones may influence the mobilization and turnover of arachidonic acid from "lipid stores" and play a key role in the control of PG production from the guinea-pig uterus.

Studies have shown that uterine phospholipase (PL) A<sub>2</sub> is stimulated by administration of steroid hormones in the hypophysectomized (Dey, Hoversland and Johnson, 1982) and ovariectomized rat (Pakrasi, Cheng and Dey, 1983). In the human endometrium, the pre-ovulatory surge of oestradiol was proposed to be the stimulus for a rapid increase in concentration of the calcium-dependent form of PLA<sub>2</sub>, which subsequently decreased as progesterone concentrations increased (Bonney, 1985; Bonney, Qizalbash and Franks, 1987). This rise in PLA<sub>2</sub> activity was shown to occur prior to maximum PG production by the endometrium at the end of the cycle (Downie, Poyser and Wunderlich, 1974; Singh, Baccarini and Zuspan, 1975).

PLA<sub>2</sub> activity has been demonstrated to increase only 1.5-fold between days 7 and 16 of the guinea-pig oestrous cycle (Downing and Poyser, 1983). Thus, an increase in PLA<sub>2</sub> activity may not be solely responsible for the 22-fold increase in uterine PGF<sub>2α</sub> production by the guinea-pig uterus which occurs between days 7 and 15 of the cycle. It has been proposed that activation of PLA<sub>2</sub>, by increasing free intracellular calcium concentrations, is probably of greater importance than the absolute activity of PLA<sub>2</sub> in controlling the supply of free arachidonic acid for PGF<sub>2α</sub>.

synthesis by the guinea-pig endometrium during the oestrous cycle (Downing and Poyser, 1983).

### **1:16 PGH synthase and the control of prostaglandin synthesis**

The enzymes that contribute to the "PG synthase complex" are present in almost all cells, and PGs have been detected in a great variety of different tissues (Bergström, 1966; Karim, Sandler and Williams, 1967). The concentrations of intracellular free fatty acids, in particular arachidonic acid, are always kept very low (Marcus, Ullman and Safier, 1969; Haye and Jacquemin, 1977) so that accumulation of PGs in tissues does not occur (Jouvenez, Nugteren, Beerthius and Van Dorp, 1970). It was demonstrated (Lands and Samuelsson, 1968; Vonkeman and Van Dorp, 1968) that fatty acids esterified in lipids were not substrates for oxidative cyclisation to PGs. Thus, a prior hydrolysis of esterified fatty acids to their non-esterified form must occur for PG synthesis to proceed. Eliasson (1959), Pace-Asciak and Wolfe (1968) and Vogt, Meyer, Kunze, Lufft and Babilli (1969) all demonstrated that PGs are quickly released when tissues are removed and allowed to stand prior to extraction, indicating that PG release and action are closely associated with PG biosynthesis in physiological conditions. It was later found that arachidonic acid itself was rapidly released from guinea-pig spleen and rat cerebral cortex after killing of the animal and manipulation of the tissue (Flower and Blackwell, 1976, Marion, Pappius and Wolfe, 1979). From the evidence, it is clear that the availability of the appropriate free fatty acid precursor to PGH synthase is of major importance in the control of PG synthesis. Previous studies on the cyclooxygenase oxygenation process in PG synthesis revealed an unusual kinetic pattern (Smith and Lands, 1972). Arachidonic acid administration to the enzyme accelerated the enzyme's activity to maximum velocity after which it gradually decreased to zero velocity. Addition of fresh enzyme resulted in further synthesis of PGs, suggesting that the enzyme was "self-limiting" or "suicidal" in behaviour. Addition of glutathione peroxidase was also shown to inhibit the cyclooxygenase activity. A prosthetic group (haem) was shown to be present within the enzyme and destroyed at a much slower rate than the enzyme (Hemler and Lands, 1980), so that the inactivation of the enzyme seemed to be due to a direct action on the enzyme and not on the haem group. Together, the evidence implies that PGG<sub>2</sub> (a hydroperoxy acid) acts as a co-factor in its own biosynthesis.

PGH synthase (PGHS-1) was originally purified from bovine (Miyamoto, Ogino, Yamamoto and Hayaishi, 1976) and ovine (Hemler, Lands and Smith, 1976; Van der Ouderaa, Buytenhek, Nugieren and Van Dorp, 1977) seminal vesicles (Samuelsson *et al.*, 1978). Cloning of human (Yokoyama and Tanabe, 1989), ovine

(DeWitt and Smith, 1989; Merlie, Fagan, Mudd and Needleman, 1987) and murine (DeWitt, El-Harith, Kraemer, Andrews, Yao and Armstrong, 1990) complementary DNA (cDNA) for PGHS-1 revealed that this protein is encoded by a 2.8-kb messenger RNA (mRNA) transcript. Recently, a novel isoform of PGHS, (PGHS-2) encoded by a distinct mRNA has been identified. An increase in PGHS activity in epithelial cells isolated from tracheal mucosa was shown to be associated with an increase in a previously undescribed 4.0-kb mRNA that hybridized to PGHS cDNA (Rosen, Birkenmeier, Raz and Holtzman, 1989) and chicken embryo fibroblasts, induced by Rous sarcoma virus, expressed a 4.1 kb mRNA encoding a 603-amino acid PGHS-like protein (Xie, Chipman, Robertson, Erikson and Simmons, 1991). A rapid induction of a 4.4-kb mRNA encoding a novel PGHS isoform has also been demonstrated in rat preovulatory follicles before ovulation (Sirois and Richards, 1992; Sirois, Simmons and Richards, 1992). The two isozymes of PGHS are denoted as PGHS-1 (Mr 69 kDa) and PGHS-2 (Mr 72 kDa), the former being a constitutive enzyme and the latter being an inducible form (Wong and Richards, 1991; Sirois and Richards, 1992).

The synthesis of PGHS-2 has been shown to be stimulated by a variety of factors including cell activation and serum (Xie *et al.*, 1991), phorbol esters (Xie *et al.*, 1991; Kujubu, Fletcher, Varnum, Lim and Herschman, 1991), chorionic gonadotrophin (CG) (Sirois and Richards, 1992; Sirois, Simmons and Richards, 1992), lipopolysaccharide (O'Sullivan, Huggins, Meade, DeWitt and McCall, 1992) and interleukin-1 $\beta$  (IL-1 $\beta$ ) (O'Banion, Winn and Young, 1992). The expression of PGHS-2 has also been shown to be selectively inhibited by dexamethasone (Kujubu and Herschman, 1992). It is possible that the inducible nature of PGHS-2 is consistent with its involvement in physiological processes. For example, the increase in PGHS that occurs in ovine fetal cotyledonary tissue at term, has been shown to be predominantly PGHS-2 (Wimsatt, Nathanielsz and Sirois, 1993). Immunohistochemical studies have also demonstrated that PGHS-2 is localized to implantation sites in newly differentiating murine uterine stromal cells at the time of blastocyst attachment, indicating a potential physiological role for PGHS-2 in the early stages of mouse implantation. This study also demonstrated that increased PG secretion by uterine stromal cells in response to interleukin-1 $\alpha$  occurs as a result of the selective expression of PGHS-2 (Jacobs, Hwang, Julian and Carson, 1994).

These studies raise the question as to whether the increase in PGF<sub>2 $\alpha$</sub>  production by the guinea-pig uterus at the end of the oestrous cycle occurs as a result of a specific increase in PGHS-2. However, in a recent study (Naderali and Poyser, in press), Western blotting analyses demonstrated that PGHS-2 was present in the

soluble extracts of guinea-pig endometrium obtained on both days 6 and 17 of the oestrous cycle. Therefore, increased  $\text{PGF}_{2\alpha}$  output at the end of the cycle does not appear to depend upon the "inducible" form of PGHS (ie PGHS-2) being synthesized specifically towards the end of the cycle in the guinea-pig.

### **1:17 The physiological role of arachidonic acid**

Arachidonic acid is an essential fatty acid i.e. it is necessary for growth and reproduction in biological systems (Burr and Burr, 1930). It is usually present in the meat and fish components of the diet, although it can also be derived from linoleic acid via chain elongation and desaturation steps (Gurr and James, 1971). PG production can be regulated by altering the distribution of arachidonic acid within the cell so that it is unavailable for release from phospholipids (Laposanta, Kaiser and Capriotti, 1988). Therefore, changes in the availability of arachidonic acid induced by diet can lead to alterations in PG synthesis. A study by Olsen, Hansen and Sorensen (1986) demonstrated that a diet rich in marine fats, which have a high content of n-3 polyunsaturated fatty acids, leads to a decrease in the synthesis of PGs of the 2-series. Women from the Faroe Islands, who have such a diet, had a longer period of gestation compared with an equivalent group of women from Denmark. Suppression of endometrial  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  synthesis following such supplementation can be explained as direct competition between these fatty acids and arachidonic acid for incorporation into membrane phospholipids. The amount of arachidonic acid available for 2-series PG synthesis will therefore be reduced. However, perinatal mortality has been shown to be higher in the Faroes than in Denmark; during 1972 - 1982, the late fetal death rates were 11.5 and 6.2 / 1000 live births and early neonatal death rates were 7.6 and 6.2 / 1000 live births, respectively (see Olsen, Hansen and Sorensen, 1986). Causes of the higher perinatal mortality in the Faroes are not clear, but may be caused by factors that are independent of birthweight and gestational age. One possibility proposed is that the increased perinatal mortality is due to side-effects of the n-3 polyunsaturated fatty acids, similar to those following obstetric use of PG synthetase inhibitors; closure of ductus arteriosus in utero, followed by neonatal pulmonary hypertension (Kierse 1981). A later study demonstrated that fish oil supplementation in the third trimester prolonged pregnancy without detrimental effects (Olsen, Hansen and Jensen, 1990).

Guinea-pig uterine cells have been shown to contain only approximately  $1\mu\text{g}$  free arachidonic acid/g uterine tissue, but as much as  $1\text{mg}$  arachidonic acid/g uterine tissue esterified in lipids (Leaver and Poyser, 1981). Therefore, arachidonic acid

esterified in lipids can be considered as "stored arachidonic acid". Arachidonic acid can be stored in both the neutral lipids and phospholipids.

### **1:18 Source of arachidonic acid for prostaglandin synthesis**

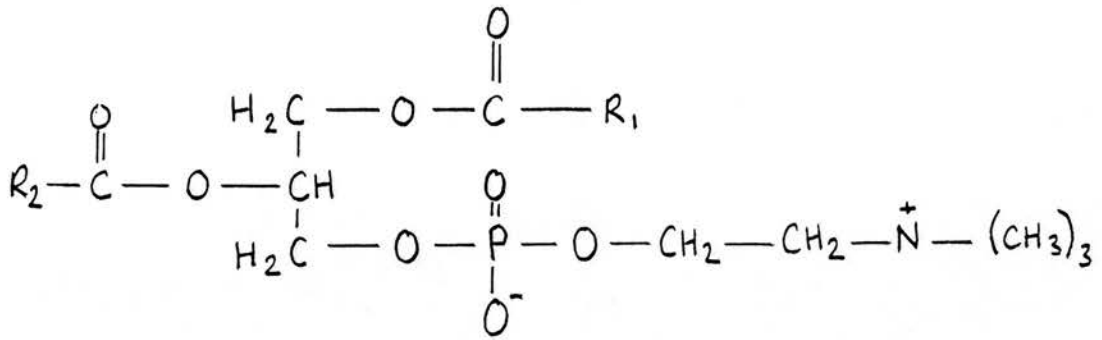
The neutral lipids are comprised of long chain fatty acids esterified to either glycerol or cholesterol, forming glycerides and cholesterol esters respectively. The triglycerides and cholesterol esters, when acted on by lipase and esterase enzymes respectively, are a potential source for supplying free arachidonic acid for the purpose of PG synthesis. In some tissues, such as the adrenal cortex or adipose tissue, where the mass of neutral lipids is equal to or greater than that of phospholipids it is possible that a large proportion of free arachidonate originates from the neutral lipids (Christ and Nugteren, 1970; Vahouny, Chamberbhan, Hodges and Treadwell, 1978). However, much evidence supports the theory that phospholipids represent the primary source of arachidonic acid for the purpose of PG synthesis. For example, Flower and Blackwell (1976) demonstrated the uptake of labelled arachidonic acid into cellular lipids on incubation with guinea-pig platelets and spleen slices. Mechanical agitation or immunological challenge of the platelets or spleen resulted in a loss of radioactivity from the phospholipid pool only, followed by an increase in the concentrations of free labelled arachidonic acid and labelled PG formation. Other studies have supported this hypothesis (Kunze and Vogt, 1971; Samuelsson, 1972; Hong and Levine, 1976; Vogt, 1978; Blackwell and Flower, 1979; Lands, 1979).

### **1:19 The phospholipids**

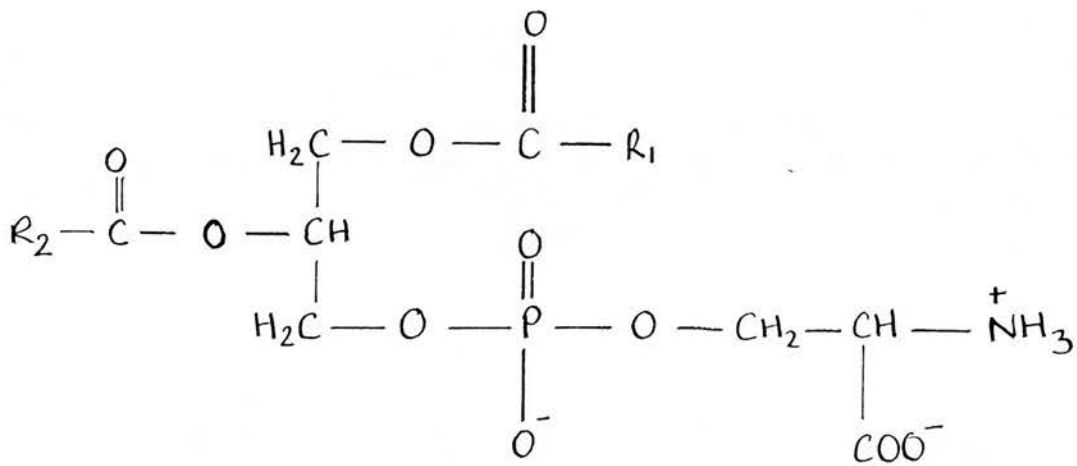
Using the stereospecific numbering (*sn*) system with the structure of glycerol represented in the Fischer projection with the C-2 hydroxyl group projecting to the left, the carbon atoms are numbered 1, 2 and 3. The phospholipids are all derived from glycerol and therefore, all have a 3 carbon backbone. The phospholipids include, amongst others, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (Sph). Glycerophospholipids contain a *sn*-glycerol 3-phosphate moiety along with two fatty acids esterified at carbons 1 and 2 (since the two primary alcohol groups of glycerol are not stereochemically identical, and in the case of phospholipids, it is usually the same hydroxyl group that is esterified to the phosphate residue). The phosphate group is joined by an ester linkage to inositol or a nitrogenous base (a phosphodiester bridge), such as choline, serine or ethanolamine from which the name of the phospholipid is derived. Figure 3 illustrates the structures of several common



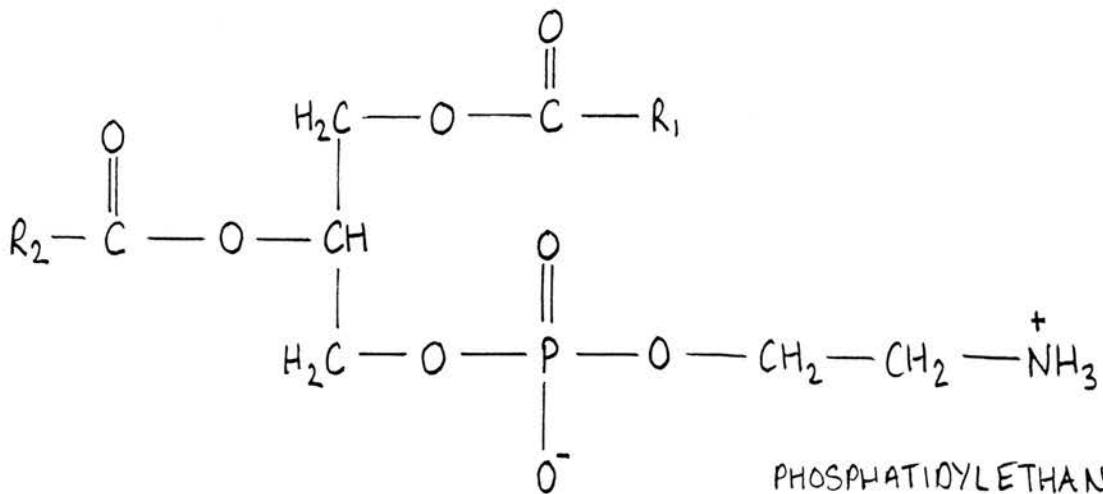
Figure 3. The structures of several common phospholipids.



PHOSPHATIDYLCHOLINE  
(LECITHIN)



PHOSPHATIDYLSERINE



PHOSPHATIDYLETHANOLAMINE  
 $\text{R}_1$  AND  $\text{R}_2$  ARE FATTY ACYL  
CHAINS

phospholipids. At physiological pH, PC and PE have no net charge and exist as dipolar zwitterions, whereas PS has a net charge of -1, causing it to be an acidic phospholipid. PE is related to PC in that trimethylation of PE produces PC. Most phospholipids contain more than one kind of fatty acid per molecule, so that a given class of phospholipids from any tissue actually represents a family of molecular types. Unsaturated fatty acids such as arachidonic acid are preferentially esterified at carbon 2 (Lands and Merck, 1963). PC and PE are synthesized by activation of the nitrogenous base, whereas PI and PS synthesis involves activation of diglyceride. With relevance to uterine PG production PC, PE and PI will be considered in more detail.

#### **1:19:1 Synthesis of phosphatidylcholine (PC)**

The major pathway for the biosynthesis of PC (lecithin) involves the sequential conversion of choline to phosphocholine, cytidine diphosphatecholine (CDP-choline) and PC. In this pathway, the phosphocholine polar head is activated using cytidine-5'-triphosphate (CTP). Free choline (a dietary requirement for most mammals) is initially phosphorylated by adenosine triphosphate (ATP), a reaction catalysed by the enzyme choline kinase, resulting in the formation of phosphocholine and adenosine diphosphate (ADP) (Wittenberg and Kornberg, 1953). The phosphocholine is then "activated" by reacting with CTP and CDP-choline and pyrophosphate (PPi), the reaction being catalysed by phosphocholine cytidyltransferase which is completely specific for cytidine nucleotides (Kennedy and Weiss, 1956). The phosphocholine moiety of CDP-choline is then transferred to the vacant primary hydroxyl group of a diglyceride (a neutral lipid), by the action of phosphocholine glyceride transferase (Weiss, Smith and Kennedy, 1958) and PC is formed and cytidine monophosphate (CMP) is released as a by-product.

PC can also be synthesized by repeated methylation of PE, by the action of phosphatidylethanolamine N-methyltransferase. The stepwise methylation of PE, with 3 moles of S-adenosylmethionine as the methyl donor, results in PC (Bremer and Greenberg, 1961). This reaction occurs in the liver in the human.

#### **1:19:2 Synthesis of phosphatidylethanolamine (PE)**

The synthesis of PE is essentially analogous to that of PC. CDP-ethanolamine and PPi are formed by the action of ethanolamine kinase and phosphoethanolamine cytidyltransferase, which is followed by the transferral of the phosphoethanolamine moiety of CDP-ethanolamine to a diglyceride resulting in the formation of PE (Kennedy and Weiss, 1956). PE can also be generated by the decarboxylation of phosphatidylserine; however, this is thought to represent only a minor pathway in PE synthesis.





### 1:19:3 Synthesis of phosphatidylinositol (PI)

Unlike PC and PE, PI synthesis involves the activation of the diglyceride, not the nitrogenous base. The initial step, catalysed by CTP-phosphatidate cytidyltransferase, involves the reaction of phosphatidic acid with CTP to form CDP-diglyceride, and PPi as a by-product. The enzyme CMP-phosphatidate:inositol phosphatidate transferase then catalyses the addition of a free inositol to the CDP-diglyceride resulting in the formation of PI (Possmayer and Strickland, 1967).

### 1:20 The phospholipases

A group of enzymes known as the phospholipases are responsible for the release of fatty acids, such as arachidonic acid, from stored phospholipids. There are a number of different phospholipase (PL) enzymes including PLA<sub>1</sub>, PLA<sub>2</sub> and PLC, each acting at a specific ester bond present in phospholipids. PLA<sub>1</sub> and PLA<sub>2</sub> cleave the acyl ester bond at the *sn*-1 and *sn*-2 position respectively, whilst PLC acts at the phosphodiester bond (Dennis, 1983). Cellular phospholipases are found in almost every type of cell and have a wide variety of functions (Waite, 1985).

#### 1:20:1 Phospholipase A<sub>1</sub>

Hydrolysis of fatty acids at the *sn*-1 position by PLA<sub>1</sub> releases 2-acyl lysophosphatides which are rapidly metabolised by lysophospholipases or reacylated to phosphatides (Lands and Merck, 1963). Since arachidonic acid is usually esterified at the *sn*-2 position the activity of PLA<sub>1</sub> is of no relevance in the release of free arachidonic acid for PG synthesis.

#### 1:20:2 Phospholipase A<sub>2</sub>

PLA<sub>2</sub> was the first phospholipase to be discovered following the observation that both pancreatic juice and cobra venom were able to hydrolyse PC (Witcoff, 1951). Using mixed preparations of PC and PE, in which *sn*-1 and *sn*-2 position fatty acids had been specified, de Haas, Daemen and Van Deenen (1962) demonstrated that PLA<sub>2</sub> activity at the *sn*-2 position resulted in the formation of a 1-acyl lysophosphatide. It was later determined that three points of attachment were necessary for hydrolysis of a fatty acid ester bond, namely at the *sn*-2 fatty acid ester linkage, oxygen alkyl group at *sn*-1 and at the hydroxyl function of the phosphoryl moiety (where Ca<sup>2+</sup> ions may act as a linkage between the phospholipase enzyme and the substrate).

Most arachidonate in mammalian cells is esterified almost exclusively in the 2-acyl position in the fatty acyl chains of glycerophospholipids and it was therefore suggested that the enzyme that was most likely to release arachidonic acid from phospholipids was phospholipase (PL) A<sub>2</sub>. The treatment of guinea-pig spleen slices

with mepacrine (an antimalarial drug which blocks PLA<sub>2</sub> activity) inhibited the release of arachidonic acid (Flower and Blackwell, 1976) indicating that PLA<sub>2</sub> was involved in the release of arachidonic acid from phospholipids. Intracellular PLA<sub>2</sub> activities have been found in almost all cells examined (Van den Bosch, 1980). PLA<sub>2</sub> perfused into guinea-pig lung (Babilli and Vogt, 1965; Vogt *et al.*, 1969) and frog intestine (Bartels, Vogt and Wille, 1968; Bartels, Kunze, Vogt and Wille, 1970) resulted in the appearance of increased amounts of PGs in the collected perfusates. Sykes, Williams and Rogers (1975) demonstrated that PLA<sub>2</sub> stimulated the synthesis of prostaglandins in homogenates of human pregnant myometrium. The administration of exogenous arachidonic acid to frog intestine (Kunze, 1970) and pregnant human myometrium (Sykes *et al.*, 1975) also resulted in a stimulation of PG synthesis suggesting that PLA<sub>2</sub> was releasing arachidonic acid from some source for PG synthesis. PLA<sub>2</sub> in plasma membranes and microsomes is totally Ca<sup>2+</sup> dependent (Newkirk and Waite, 1973) whereas other lipolytic enzymes do not require Ca<sup>2+</sup> and are therefore not inhibited by EDTA (ethylene diamine tetra-acetic acid) (McMurray and Magee, 1972). PG synthesis in homogenates of bovine seminal vesicles is markedly increased by Ca<sup>2+</sup> and inhibited by EDTA (Kunze, Bohn and Vogt, 1974) providing further evidence for a role of PLA<sub>2</sub> in PG synthesis.

More recent studies have demonstrated that the release of arachidonic acid from membrane phospholipids occurs via receptor activation of PLA<sub>2</sub> by a G-protein. In various tissues, including neutrophils, fibroblasts and sensory neurones, PLA<sub>2</sub> is activated by a G-protein which is inhibited by pertussis toxin (ADP-ribosylates G-proteins) (Burch, Luini and Axelrod, 1986; Burgoyne, Cheek and O'Sullivan, 1987; Axelrod, Burch and Jelsema, 1988; Wang, Kester and Dumm, 1988; Nakashima, Nagata, Ueeda and Nozawa, 1988). Fluoride (an activator of G-proteins) stimulates PGI<sub>2</sub> synthesis by the rat aorta (Jeremy and Dandona, 1988). Many receptors that are known to activate PLA<sub>2</sub> are also coupled to PLC. Since the products of PLC activation may in theory stimulate PLA<sub>2</sub>, various studies were performed to clarify whether sequential activation, as opposed to parallel activation of the two lipases, was occurring. Pertussis toxin was shown to selectively inhibit PLA<sub>2</sub> activation without impairing PLC activation and neomycin (a PLC inhibitor) selectively inhibited PLC without inhibiting PLA<sub>2</sub> (Burch *et al.*, 1986) supporting parallel activation of the two lipases.

Cockcroft, Nielson and Stutchfield (1991) proposed that PLA<sub>2</sub> activation is under dual regulation in human neutrophils; a G-protein-dependent pathway and a Ca<sup>2+</sup> / PKC-regulated pathway. Lipocortins are Ca<sup>2+</sup> / lipid-binding proteins that block the access of PLA<sub>2</sub> to the phospholipid substrate. Touqui, Rothut, Shaw, Fradin,

Vargaftig and Russo-Marie (1986) proposed a model of PLA<sub>2</sub> activation in human platelets whereby phosphorylation of lipocortin removes the inhibitory constraint from the PLA<sub>2</sub>, thus allowing activation. Lipocortins have also been shown to be substrates for PKC in human neutrophils (Stoehr, Smolen and Suchard, 1990). Glucocorticoid hormones stimulate the production of lipocortin in many tissues, therefore inhibiting PLA<sub>2</sub> activity and resulting in decreased PG synthesis and release (Flower, 1986). In the guinea-pig, endometrial PLA<sub>2</sub> activity does not appear to be restrained by a PKC-inactivated lipocortin, since glucocorticoids had no effect on PG synthesis from the guinea-pig endometrium in culture (Riley and Poyser, 1987b).

In the guinea-pig endometrium, PGF<sub>2α</sub> synthesis is not regulated by a toxin-sensitive G-protein, but may be regulated by a fluoride-sensitive G-protein (Leckie and Poyser, 1990a). This fluoride-mediated stimulation of prostaglandin output from the guinea-pig uterus may be due to the activation of a G-protein resulting in the mobilization of intracellular calcium (by a mechanism which does not involve the activation of PLC or calmodulin) which in turn, activates PLA<sub>2</sub> (Leckie and Poyser, 1990b).

PLA<sub>2</sub> enzymes are found both extracellularly and intracellularly. The different kinds of mammalian PLA<sub>2</sub> (from Mukherjee, Miele and Pattabiraman, 1994) enzymes are listed below:-

**Type I - Low molecular weight (14 kDa) PLA<sub>2</sub>s**

Group I - (secretory and cytosolic): pancreas, lung, spleen, gastric mucosa, sperm, serum.

Group II - (secretory and membrane-associated): synovial fluid, placenta, platelets, neutrophils, mesangium, astrocytes, hepatocytes, seminal fluid, peritoneal fluid, serum.

**Type II - Ca<sup>2+</sup>-independent PLA<sub>2</sub>s**

40 kDa (microsomal): plasmalogen-selective; myocardium.

58 kDa (cytosolic): dimeric, PE-selective; platelets.

**Type III - cPLA<sub>2</sub>**

85 kDa (cytosolic) regulated by Ca<sup>2+</sup>, G-proteins and phosphorylation; neutrophils, platelets, macrophage-like cells, liver, brain.

**Type IV- Lysosomal PLA<sub>2</sub>s**

Acidic pH optima; ubiquitous

The most-thoroughly characterized PLA<sub>2</sub>s have been small molecular weight (14 kDa) enzymes, present in many tissues and extracellular fluids, referred to as "Type I" or "secretory" PLA<sub>2</sub>s. These have a high disulphide bond content, require millimolar concentrations of Ca<sup>2+</sup> for catalysis and exhibit little or no fatty acyl specificity. Two

groups of Type I PLA<sub>2</sub>s have been isolated (see Mukherjee *et al.*, 1994). Group I are secreted as zymogens by the pancreas, but have also been isolated from other organs including the lung, spleen and gastric mucosa. In the latter two organs, the enzyme is intracellular. A non-pancreatic, Group I PLA<sub>2</sub> is also present in human spermatozoa, distinct from the seminal fluid Group II enzyme (Cordella-Miele, Miele and Mukherjee, 1993). Group II PLA<sub>2</sub> has been isolated from human synovial fluid, and is also present in the plasma where it is significantly elevated in numerous pathologic disorders (see Rosenthal, Rzigalinski, Blackmore and Franson, 1995). It has also been shown to be present in several tissues as secretory and cell surface associated enzymes (eg. placenta, platelets, neutrophils and peritoneum). In platelets and mast cells, Group II PLA<sub>2</sub> appears to be associated with intracellular granules and secreted during cellular degranulation (Kudo, Murakami, Hara and Inoue, 1993). In humans, it appears that there is a single gene for Group II PLA<sub>2</sub> (Mayer and Marshall, 1993). An ischemia-induced myocardial 40 kDa, Ca<sup>2+</sup>-independent, microsomal PLA<sub>2</sub>, that preferentially hydrolyzes *sn*-2 arachidonyl plasmalogen substrates has also been described (Hazen, Stuppy and Gross, 1990; Hazen, Ford and Gross, 1991). The isolation of a dimeric 58 kDa Ca<sup>2+</sup>-independent PLA<sub>2</sub> from sheep platelets and cloning of its cDNA have also been reported (Zupan, Steffens, Berry, Landt and Gross, 1992). This enzyme (also referred to as "14-3-3 protein") is found in the cytosol and selectively hydrolyzes 2-arachidonyl PE (Zupan *et al.*, 1992).

In addition to the well known Type I or secretory PLA<sub>2</sub>s (sPLA<sub>2</sub>) with a low molecular weight (14 kDa), a new form of PLA<sub>2</sub> ("cytosolic" or "cPLA<sub>2</sub>") has been demonstrated (also referred to as "Type III PLA<sub>2</sub>"). This PLA<sub>2</sub> has a high molecular weight (85 kDa), can translocate from cytosol to cell membrane in the presence of micromolar concentrations of Ca<sup>2+</sup>, and is linked to membrane receptors (Clark, Lin, Kriz, Ramesha, Sultzman, Lin, Milona and Knopf, 1991). cPLA<sub>2</sub> lacks disulphide bonds and shows no significant sequence similarity with any other PLA<sub>2</sub>. The enzyme exhibits a marked specificity for *sn*-2 arachidonyl phospholipids (Mayer and Marshall, 1993). Other PLA<sub>2</sub> activities have been demonstrated in lysosomes having low pH optima and which are Ca<sup>2+</sup>-independent (see Mukherjee *et al.*, 1994).

Both cytosolic 85 kDa PLA<sub>2</sub> (cPLA<sub>2</sub>) and secretory non-pancreatic Group II 14 kDa PLA<sub>2</sub> (sPLA<sub>2</sub>) may contribute to arachidonate mobilization and therefore PG synthesis. One difficulty in distinguishing the relative contributions of different PLA<sub>2</sub>s in cellular systems is the lack of selective, membrane permeant PLA<sub>2</sub> inhibitors. For example, PLA<sub>2</sub> inhibitors such as PGBx, PX-52 and aristolochic acid have been shown to have similar IC<sub>50</sub>s *in vitro* for both 85 and 14 kDa PLA<sub>2</sub>s (Franson and

Rosenthal, 1994). Further studies are required to fully elucidate the mechanisms which regulate activation of cellular phospholipases and subsequent synthesis.

### **1:20:3 Phospholipase C**

The action of PLC on PI also provides a mechanism by which arachidonic acid is released, and this mechanism has been mainly studied in thrombin- or collagen-stimulated platelets. PLC releases 1,2-diacylglycerol (DAG) (Rittenhouse-Simmons, 1979) from which arachidonic acid may be cleaved directly by the action of DAG and monoacylglycerol lipases (Flint *et al.*, 1986a). It is doubtful that PLC activity is related to uterine PG release in the guinea-pig since PI contains less than 10 % of the total arachidonic acid present in the guinea-pig uterus (Leaver and Poyser, 1981) and no change in PI turnover was reported during the oestrous cycle (Ning and Poyser, 1984). Phorbol 12-myristate 13-acetate (PMA, a PKC activator) had no effect on PG output from cultured guinea-pig endometrial tissue (Riley and Poyser, 1987a) or from superfused uterus *in vitro* (Poyser, 1987a) on either day 7 or 15 of the oestrous cycle. Both PLC and PLA<sub>2</sub> stimulated PG outputs from day 7 and 15 superfused guinea-pig uterus *in vitro*. However, PLA<sub>2</sub> released a pattern of PGs similar to those released by calcium (mainly PGF<sub>2α</sub> at the time of luteolysis), whereas PLC released a significantly greater amount of PGE<sub>2</sub> (Poyser, 1987a). It was therefore concluded that stimulation of endogenous PLA<sub>2</sub> by calcium rather than stimulation of endogenous PLC was involved in the release of uterine arachidonic acid for the purpose of PGF<sub>2α</sub> synthesis in the guinea-pig at the time of luteolysis (Poyser, 1987a).

Agonists that mimic the effects of DAG and IP<sub>3</sub> (second messengers generated by PLC) have been shown to stimulate the release of PGF<sub>2α</sub> by ovine and bovine endometrial tissue *in vitro* (Silvia and Homanics, 1988; Lafrance and Goff, 1990; Silvia *et al.*, 1994). However, further studies demonstrated that considerable PGF<sub>2α</sub> release occurred with little or no detectable increase in PLC (Raw and Silvia, 1991; Mirando *et al.*, 1993; Silvia and Raw, 1993a). It appears that PGF<sub>2α</sub> release from ovine endometrial tissue is mediated through the activation of PLA<sub>2</sub> rather than through activation of PLC (Lee and Silvia, 1994).

### **1:21 Activity of PLA<sub>2</sub> in the uterus**

Both exogenous PLA<sub>2</sub> and melittin (a stimulator of PLA<sub>2</sub>) stimulated uterine PGF<sub>2α</sub> release (Poyser, 1987a; Johnson and Poyser, 1991) whereas p-bromophenacyl bromide (an inhibitor of PLA<sub>2</sub>) suppressed the basal synthesis of PGF<sub>2α</sub> by homogenates of guinea-pig uterine tissue (Mitchell, Poyser and Wilson, 1977). An increase in guinea-pig uterine PLA<sub>2</sub> activity has also been shown to occur towards the



end of the cycle when PG secretion is maximal (Downing and Poyser, 1983). PLA<sub>2</sub> activity was investigated in guinea-pig endometrium on days 7 and 15 of the oestrous cycle (days of low and high PG production, respectively) and was found to be present in both microsomal and supernatant fractions with 90 % activity present in the microsomal fraction (presumably membrane-bound). The PLA<sub>2</sub> activity in both fractions was optimal at pH 8.0 requiring 7 mM and 2 mM Ca<sup>2+</sup> in the microsomal and supernatant fractions respectively. The microsomal PLA<sub>2</sub> activity appeared to require an increase in free intracellular Ca<sup>2+</sup> concentration for stimulation of maximal enzymal activity (Downing and Poyser, 1983). Several studies have shown that the guinea-pig endometrium has a potential for arachidonic acid release that far exceeds the observed PG production (Downing and Poyser, 1983; Poyser and Brydon, 1983; Poyser and MauleWalker, 1979). It was therefore proposed that the activation of PLA<sub>2</sub> could be partly responsible for regulating uterine PG production during the oestrous cycle. Activation of PLA<sub>2</sub> also appears to play an important role in regulating the uterine secretion of PGs in the sheep (Lee and Silvia, 1994) and in the human (Bonney, Beesley and Franks, 1991). A PLA<sub>2</sub>-stimulating protein (PLAP), which is antigenically and functionally related to the PLA<sub>2</sub>-stimulating peptide melittin, has been isolated from various cultured cell lines, and is an "intracellular messenger" in the stimulation of PG synthesis (Clark, Conway, Shorr and Crooke, 1987; Clark, Chen, Crooke and Bomalaski, 1988). Further work has identified PLAP as a new member of the  $\beta$ -transducin (G $\beta$ ) superfamily, strengthening the evidence that G-proteins play a key role in the activation of PLA<sub>2</sub> (Peitsch, Borner and Tschopp, 1993). However, it is unclear whether PLAP is produced by the guinea-pig endometrium (Leckie and Poyser, 1993).

The concentration of lipocortin has been shown to increase during pregnancy in the rat (Kaetzel, Hazarika and Dedman, 1989). In the sheep, PLA<sub>2</sub> activity was shown to decrease by 58 % in day 16 pregnant endometrium compared to day 16 nonpregnant endometrium. This inhibition appeared to be due to a PLA<sub>2</sub> inhibitor present in the pregnant uterus which possibly acted as a lipocortin (Tamby, Charpigny, Reinaud and Martal, 1993). It has been proposed that a PLA<sub>2</sub> inhibitor (possibly a lipocortin) is secreted from the early pregnant ovine endometrium which may contribute to the changes in PG synthesis during early pregnancy (Tamby *et al.*, 1993).

### **1:22 Role of calcium in prostaglandin synthesis**

Administration of A23187 results in the stimulation of PG production in several different tissues (Knapp, Oelz, Roberts, Sweetman, Oats and Reep, 1977)



whereas treatment with EDTA prevents this stimulatory effect (Kunze *et al.*, 1974). Addition of exogenous arachidonic acid to cultured guinea-pig endometrium lead to a rise in PG production comparable to that seen following treatment with A23187 (Leaver and Seawright, 1982). The stimulatory action of A23187 was abolished when a  $\text{Ca}^{2+}$ -free medium was used confirming that A23187 was acting as a calcium ionophore and not by some other unspecified mechanism (Poyser, 1984b). This lead to the suggestion that a rise in the intracellular free  $\text{Ca}^{2+}$  concentration may regulate the availability of PG precursor. This mechanism of action was further confirmed by the observation that treatment with the intracellular calcium antagonist TMB-8, inhibited the A23187-induced increase in  $\text{PGF}_{2\alpha}$  (and 6-keto- $\text{PGF}_{1\alpha}$ ) from day 15 superfused guinea-pig uterus (Poyser, 1985b). Maximal stimulatory calcium concentrations are in the mM range, whereas intracellular calcium concentrations exist in the  $\mu\text{M}$  range. However, a small increase in calcium concentration in the  $\mu\text{M}$  range may be sufficient to stimulate  $\text{PLA}_2$  to an adequate level resulting in increased PG synthesis. Although calcium may activate  $\text{PLA}_2$  directly, it may also be acting via a mediator.

### **1:23 Role of calmodulin in prostaglandin synthesis**

Calmodulin is a calcium binding protein (17 kDa) possessing a single polypeptide chain with 2 high and 2 low affinity calcium ion binding sites. Binding of  $\text{Ca}^{2+}$  to the lower affinity binding sites causes a conformational change in the protein revealing a hydrophobic area that can then interact with a protein that it controls. Each  $\text{Ca}^{2+}$  binding site consists of a helix-loop-helix structure,  $\text{Ca}^{2+}$  being bound in the loop connecting the helices. Calmodulin was demonstrated to play an important role in the activation and regulation of many  $\text{Ca}^{2+}$  dependent processes in eukaryote cells including several enzyme activities (Cheung, 1980).  $\text{PLA}_2$  was shown to be both  $\text{Ca}^{2+}$  and calmodulin dependent (Wong and Cheung, 1979; Moskowitz, Andres, Silva, Shapiro, Schook and Puszkun, 1985). The calmodulin inhibitor, trifluoperazine (TFP) which acts by binding to the hydrophobic domain on the surface of the calmodulin and therefore blocks interaction of calmodulin with its surface receptor protein (Andersson, Drakenberg, Thulin and Forsen, 1983), abolished the release of arachidonic acid and synthesis of PGs induced by A23187, vasopressin, NaCl and mannitol in rat renal medulla (Craven and DuRubertis, 1983). TFP was also shown to inhibit  $\text{PLA}_2$  and therefore arachidonic acid release from platelets (Walenga, Opas and Feinstein, 1981) and PG production from dispersed human amnion cells (Olson, Challis, Opavsky, Smeijja, Kramar and Skinner, 1985). A role for calmodulin in the control of  $\text{PGF}_{2\alpha}$  synthesis and therefore  $\text{PLA}_2$  activity was also demonstrated in the

guinea-pig uterus when A23187-stimulated  $\text{PGF}_{2\alpha}$  output was prevented by TFP and W-7 (another calmodulin antagonist) in superfused guinea-pig uterus *in vitro* (Poyser, 1985a,b). Oestradiol treatment increased the concentration of calmodulin in rat (Flandroy, Cheung and Steiner, 1983) and rabbit (Matsui, Higashi, Fukunaga, Miyazaki, Maeyama and Miyamoto, 1983) uterine tissue. Therefore, calmodulin may also contribute to the regulation of  $\text{PGF}_{2\alpha}$  secretion in these species.

## **1:24 Arachidonic acid turnover and prostaglandin release**

### **1:24:1 Introduction**

It is now well established that in most tissues, such as the guinea-pig uterus, the synthesis of PGs is limited by the availability of their precursor, arachidonic acid, which must be liberated from esterified stores. Since the majority of arachidonate is esterified in the fatty acyl chains of glycerophospholipids, almost exclusively in the 2-acyl position, it was previously suggested that the phospholipases are responsible for the control of free arachidonic acid levels (van Dorp *et al.*, 1964; Bergström *et al.*, 1964; Lands and Samuelsson, 1968; Vonkeman and van Dorp, 1968; Vogt, 1978; Kroener, Peskar, Fischer and Ferber, 1981). However, free fatty acid level in a tissue actually represents a balance between the liberation of the acid by hydrolysis and its re-esterification into complex lipids by acyl transferase reactions (Trotter and Ferber, 1981).

Studies on the guinea-pig uterus have demonstrated that 93 % of the total arachidonic acid is esterified to phospholipids, 80 % of which is bound to PC and PE. A significant decrease in arachidonic acid bound to PC and an apparent decrease from PE between days 7 and 15 of the oestrous cycle were observed in the guinea-pig uterus (Leaver and Poyser, 1981). During 24 h tissue culture of guinea-pig endometrial tissue, significantly greater quantities of labelled arachidonic (but not oleic acid) were incorporated into phospholipids (particularly PC and PE) on day 15 compared to day 7 of the oestrous cycle (Ning, Leaver and Poyser, 1983). In 1984, Ning and Poyser demonstrated that PC was the major phospholipid present in the guinea-pig uterus with lesser amounts of PE, PI and PS. However, no significant changes in the actual amounts of endometrial phospholipids were observed during the oestrous cycle. Therefore, the observed increased incorporation of arachidonic acid into endometrial tissue of mid- and late-cycle guinea-pigs cannot be due to a change in phospholipid content. This is further clarified by measurement of the *de novo* synthesis of the phospholipids, which remains unchanged between the two days in question (Ning and Poyser, 1984). Incorporation of arachidonic acid into phospholipids appears to be achieved by its acylation to a glycerolysophospholipid,

such as lysophosphatidylcholine, rather than during *de novo* synthesis of phospholipids (van den Bosch, 1974). This mechanism of arachidonic acid incorporation into phospholipids has been previously suggested by Hill and Lands (1968) and Yamashita, Hosaka and Numa (1973).

The two phospholipases (PL), PLA<sub>1</sub> and PLA<sub>2</sub>, exist in many tissues, and play an important role in the formation of specific phospholipid structures containing the appropriate fatty acids in the *sn*-1 and *sn*-2 positions. Many fatty acyl-CoA transferases and phospholipid synthesizing enzymes lack the specificity required to account for the asymmetric position or distribution of fatty acids found in many tissue phospholipids. The fatty acids found in the *sn*-1 and *sn*-2 positions of the various phospholipids are often not the same ones originally transferred to the glycerol backbone in the initial acyl-transferase reactions of the phospholipid biosynthetic pathways, indicating that a certain amount of fatty acid exchange between phospholipids occurs after phospholipid synthesis. PLA<sub>1</sub> and PLA<sub>2</sub> catalyse the reactions shown in Figure 4. The products formed are called lysophosphatides. If it becomes necessary for a cell to remove some undesired fatty acid, such as stearic acid from the *sn*-2 position of PC, and replace it by a more unsaturated one such as arachidonic acid, then this can be accomplished by the action of PLA<sub>2</sub> followed by a reacylation step.

In the guinea-pig uterus a refractoriness is observed following repeated stimulation with PLA<sub>2</sub>, but not with arachidonic acid which always induces maximum PG synthesis (Poyser, 1991). It may be that, in the guinea-pig, the refractoriness of uterine PGF<sub>2α</sub> synthesis is a consequence of the depletion of the arachidonic acid pool which is esterified to phospholipids. This arachidonic acid pool would appear to be readily releasable for prostaglandin synthesis, but requires 3-5 h to be refilled. However, in this study extracellular Type I (Group I) PLA<sub>2</sub> from *Naja Naja* venom was used. The PLA<sub>2</sub>(s) responsible for arachidonic acid mobilization for PG synthesis in the guinea-pig endometrium has not been defined, but it is known that an increase in PGF<sub>2α</sub> synthesis requires intracellular Ca<sup>2+</sup> (Poyser, 1985 a,b). Type III PLA<sub>2</sub> (cPLA<sub>2</sub>) (85 kDa) is found in the cytosol, is regulated by Ca<sup>2+</sup> and G-proteins and may therefore be the PLA<sub>2</sub>, or one of the PLA<sub>2</sub>s, responsible for PG synthesis in the guinea-pig endometrium. The A23187-stimulated increase in PGF<sub>2α</sub> output from the superfused guinea-pig uterus *in vitro* is dependent on intracellular Ca<sup>2+</sup> (Poyser, 1985 b), presumably resulting in a stimulation of PLA<sub>2</sub> and therefore PG synthesis. However, there is no cross-refractoriness between PLA<sub>2</sub> (from *Naja Naja* venom) and A23187 as regards their stimulatory effects on PG production by the guinea-pig uterus (Poyser and Ferguson, 1993), suggesting that the intracellular processes

involved in the stimulation of uterine PG output by PLA<sub>2</sub> from *Naja Naja* venom and A23187 are different. It is possible that extracellular PLA<sub>2</sub> from *Naja Naja* venom used in these studies acts in a different way to that of guinea-pig endometrial PLA<sub>2</sub> *in vivo* stimulated by A23187; this would explain the lack of cross-refractoriness. The catalytic mechanisms of other PLA<sub>2</sub>s have not yet been clarified in detail, but do appear to be different from that of low molecular weight PLA<sub>2</sub>s (Type I, such as PLA<sub>2</sub> from *Naja Naja* venom) (Mayer and Marshall, 1993).

The mechanisms that control arachidonic acid incorporation into phospholipids may be as essential as those which control arachidonic acid release from phospholipid stores in the regulation of endometrial PGF<sub>2α</sub> synthesis. Indeed, several studies have demonstrated increased arachidonic acid incorporation into endometrial phospholipids at the time of increased uterine PG production in the guinea-pig (Ning, Leaver and Poyser, 1983) and human (Downing, Hutchon and Poyser, 1983; Ishihara, Tsutsumi and Mizuno, 1986). The two enzymes involved in fatty acid incorporation into phospholipids are acyl-CoA synthetase and acyl-CoA:lysophospholipid acyltransferase. In this pathway (Lands and Crawford, 1976) acyl moieties of glycerophospholipids are initially cleaved by PLA<sub>2</sub> and then reacylated by acyl-CoA:lysophospholipid acyltransferase, using acyl-CoA formed from free fatty acids by the action of acyl-CoA synthetase. This reacylation system may be designated as the CoA- and ATP-dependent reacylation system and is shown in Figure 4.

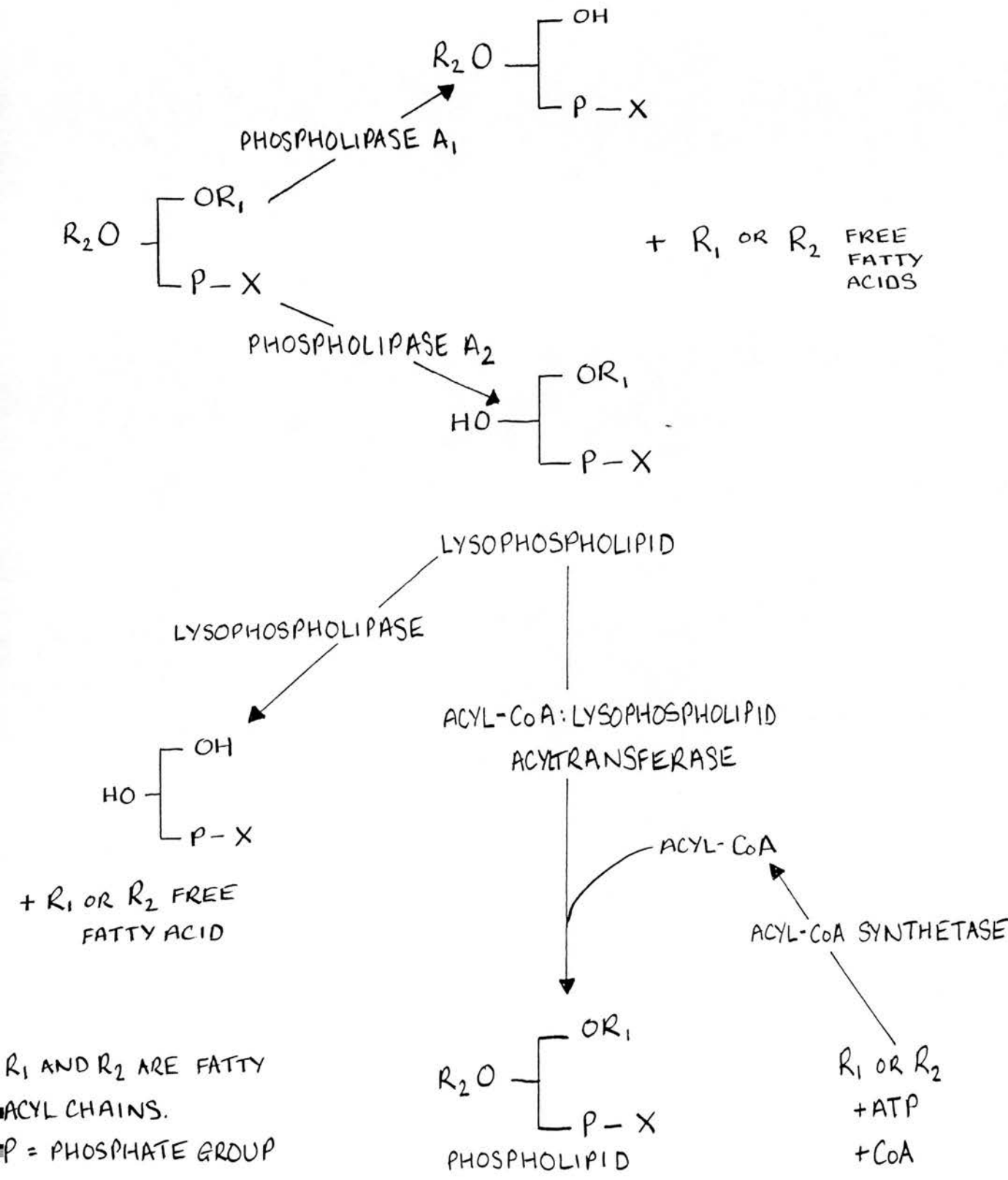
Arachidonic acid turnover between membrane phospholipids may therefore provide an indirect method by which the concentration of free arachidonic acid available for PG synthesis is controlled. Greater amounts of arachidonic acid could be selectively esterified into particular phospholipids that are more likely to undergo hydrolysis by PLA<sub>2</sub> thereby resulting in greater quantities of arachidonic acid being released and available for PG synthesis.

#### **1:24:2 Acyl-CoA synthetase**

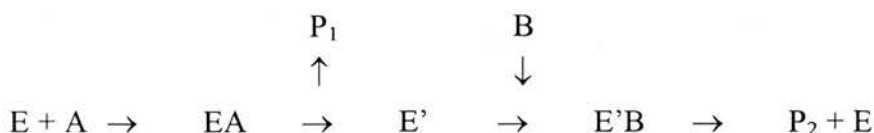
Long chain acyl-CoA synthetase was first demonstrated by Kornberg and Pricer (1953) and later purified (Tanaka, Hosaka, Hoshimaru and Numa, 1979) from rat liver mitochondria and microsomes.

Most enzymes utilize more than one substrate, or they act upon one substrate plus a coenzyme and generate one or more products. Mechanistically, multisubstrate enzyme reactions are divided into two major categories, ping-pong or sequential. The ping-pong mechanism can be diagrammatically outlined as follows:

Figure 4. The de- and re-acylation of glycerophospholipids, where X represents the polar head group of the phospholipid.



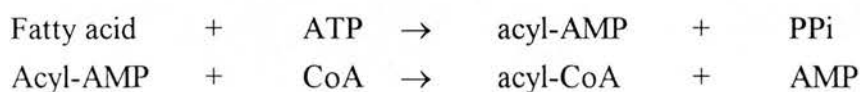




in which substrate A reacts with the enzyme (E) to produce product one ( $P_1$ ), which is released before the second substrate B will bind to the modified enzyme ( $E'$ ). Substrate B is then converted to product two ( $P_2$ ) and the enzyme regenerated.

In the sequential mechanism, if the two substrates A and B can bind in any order, it is a random mechanism. However, if the binding of A is required before B can be bound, then it is an ordered mechanism. In either case the reaction is bimolecular i.e. both A and B must be bound before reaction occurs.

The enzymatic reaction of acyl-CoA synthetase was concluded by Berg (1956) to occur via a ping-pong mechanism. As a first step, the enzyme, fatty acid and ATP form a complex, leading to the generation of an acyl-AMP enzyme complex. This complex reacts with CoA to liberate acyl-CoA and AMP.



Long chain fatty acyl-CoA synthetase has also been purified from *Candida lipolytica* (Hosaka, Mishina, Tanaka, Kamiryo and Numa, 1979) and *E.coli* (Kameda, Suzuki and Imai, 1985). cDNA encoding the long chain acyl-CoA synthetase of rat liver microsomes has been isolated and sequenced (Suzuki, Kawarabayasi, Kondo, Abe, Nishikawa, Kimura, Hashimoto and Yamamoto, 1990). Acyl-CoA synthetase was predicted to contain 699 amino acid residues with a calculated molecular weight of 78,177. The messenger RNA of the long chain acyl-CoA synthetase is expressed in liver, heart, epididymal adipose tissue and to a much lesser extent (about 10 %) in brain, small intestine and lung. The level of the mRNA is increased 7- to 8-fold in rat liver by feeding a diet high in carbohydrate or fat, in accordance with the physiological role of the enzyme in fatty acid metabolism.

It has been proposed that acyl-CoA synthetase activity is responsible for fatty acid uptake into cells i.e. transmembrane transport of fatty acids. It is widely believed that free fatty acids enter the cytosolic compartment of cells passively by diffusion through lipid bilayer membranes. Two mechanisms of fatty acid uptake into the cell across the plasma membrane involving acyl-CoA synthetase have been suggested (Neufeld, Wilson, Sprecher and Majerus, 1983).



- i. The acyl-CoA synthetase enzymes convert fatty acids that have diffused into the cell across the plasma membrane to polar fatty acyl-CoA metabolites. These are unable to diffuse through lipid bilayers and are rapidly esterified into more complex lipids.
- ii. The acyl-CoA synthetase enzymes reduce the mass of cellular free fatty acid by converting it to fatty acyl-CoA creating a gradient, which via mass action, brings more free fatty acid from exogenous pools into the cell through the plasma membrane.

Neufeld *et al.* (1983) have demonstrated two distinct fatty acid uptake systems (defined as esterification of fatty acids into phospholipids) in human platelets, namely a high affinity system specific for eicosanoid precursor fatty acids such as arachidonic acid, and a low affinity system specific for non-eicosanoid precursor fatty acids.

Interestingly, while there is an increase in the uptake of arachidonic acid into phospholipids by day 15 guinea-pig endometrium, there is not an increase in the uptake of oleic acid (Ning *et al.*, 1983) suggesting that there may be an acyl-CoA synthetase present in the endometrium specific for arachidonic acid. Such an arachidonic acid specific acyl-CoA synthetase has been demonstrated in a wide variety of tissues such as human platelets (Wilson, Prescott and Majerus, 1982), brain (Reddy and Bazan, 1983), murine T lymphocytes (Taylor, Sprecher and Russell, 1985) and rabbit coronary microvessel endothelial cells (Gerritsen and Perry, 1990). This enzyme, known as arachidonyl-CoA synthetase (ACS), has been shown to be a protein distinct from the previously described nonspecific long chain acyl-CoA synthetase (Laposanta, Reich and Majerus, 1985), although other workers have disputed this finding (Bakken, Farstad and Holmsen, 1991). Several lines of evidence suggest that ACS plays an essential role in eicosanoid production. In one study, a mutant cell line derived from HSDM<sub>1</sub>C<sub>1</sub> fibrosarcoma which contained no ACS, was found to have a significantly decreased PG production (Neufeld, Bross and Majerus, 1984). Furthermore, ACS was shown to recognise arachidonate and only those polyunsaturated acids that are converted by cyclooxygenase and lipoxygenase to eicosanoids. The enzyme apparently "counts" double bonds from the carboxy terminus (Neufeld, Sprecher, Evans and Majerus, 1984). If such enzymes are present in the guinea-pig endometrium, a specific increase in the activity of ACS towards the end of the oestrous cycle could explain the specific uptake of arachidonic acid, but not oleic acid, into phospholipids which occurs at that time.

### **1:24:3 Acyl-CoA:lysophospholipid acyltransferase**

The difference in the turnover rates of fatty acyl moieties and glycerol moieties led to the discovery of the presence of enzymes known as acyl-CoA:lysophospholipid acyltransferases in rat liver (Lands, 1958, 1960) which catalyse the reaction of acyl-CoA with lysophospholipid liberating phospholipid. The enzyme system acylating the

1-position of 2-acylglycerolphosphorylcholine has been shown to be more active with saturated acyl-CoAs, whereas the one acylating the 2-position is more active with unsaturated acyl-CoAs (Lands and Hart, 1965). These findings originally emphasised the importance of these enzyme systems in the selective placement of different fatty acids between the two positions of glycerophospholipids. The specificity of the fatty acid transfer to lysophosphatides was shown to depend exclusively on the nature of the transferred fatty acid and not on the composition of the lysophosphatides in the microsomal membranes of stimulated rabbit lymphocytes (Ferber and Resch, 1973) supporting the previous findings of Lands and Hart (1965), Stoffel, Schiefer and Wolf (1966), Brandt and Lands (1967), Stoffel, de Thomas and Schiefer (1967), van den Bosch, van Golde, Eibl and van Deenen (1967), van den Bosch, van Golde, Slotboom and van Deenen (1968), and Sarzala, van Golde, Kruffy and van Deenen (1970). A later study led to the proposition that different enzymes or different sites on a single enzyme existed in rat liver microsomes for the transfers of different acyl-CoAs (Okuyama, Yamada and Ikezawa, 1975), and that the preference of microsomal acyltransferase for arachidonyl-CoA over other acyl-CoAs was greatly enhanced if the concentration of the acyl acceptor was lowered (Holub, MacNaughton and Piekarski, 1979). It was demonstrated that a CoA-dependent transfer of fatty acyl chains between phospholipids could provide a means of achieving turnover of phospholipid acyl chains in homogenates of murine thymocytes. The maximal activity of the enzyme acylCoA:lysophospholipid acyltransferase (ACLS) (extracted from rat liver microsomes) with arachidonyl-CoA was found to occur at pH 8.0 (Trotter *et al.*, 1982) indicating that esterification by this pathway is increasingly favoured with more unsaturated fatty acids such as arachidonic acid (Hasegawa-Sakai and Ohno, 1980).

Polyunsaturated fatty acids, such as arachidonic acid, are liberated in many tissues by the action of PLA<sub>2</sub>. Cleavage of phospholipids by this enzyme not only generates free fatty acids, but also lysophosphatides, which are very toxic due to their detergent-like properties (Weltzien, 1979). In order to protect the cell from such toxic action, lysophosphatides must be rapidly degraded or reacylated. The latter reaction was first described by Hill and Lands (1968), and is mediated by the enzyme ACLS (also known as lysophosphatide acyltransferase). In many cells ACLS activity far exceeds that of PLA<sub>2</sub>, compatible with its role as a "safety" enzyme (Van den Bosch, 1974). It was therefore proposed that the cellular levels of free fatty acids may be controlled by the activity of the reacylating enzymes, mainly ACLS, rather than by PLA<sub>2</sub> activity (Irvine, 1982). Indeed, this has been demonstrated in bone marrow-derived macrophages (Kroener *et al.*, 1981), lung fibroblasts (Hunter, Burstein and Sedor, 1984), rat peritoneal-derived macrophages and human platelets (Goppelt-

Struebe, Kroener, Hausmann, Gema and Resch, 1986). Also, a unique cardiac cytosolic acyltransferase from rabbit heart has been demonstrated to be selective for fatty acids that are substrates for the cyclooxygenase or lipoxygenase pathway (Needleman, Wyche, Sprecher, Elliott and Evers, 1985). Therefore, selective incorporation of arachidonic acid into cardiac phospholipids can possibly be achieved by the unique cytosolic acyltransferase, whereas platelets utilise an arachidonate-specific ACS (Wilson *et al.*, 1982).

Various reports have demonstrated that ACLS can also operate in reverse, such as in mouse lymphocytes and macrophages (Trotter, Flesch, Schmidt and Ferber, 1982), human platelets (Kramer and Deykin, 1983) and guinea-pig alveolar macrophages (Nijssen and van den Bosch, 1986). This pathway was first described by Irvine and Dawson (1979) and involves the CoA-dependent and ATP-independent breakdown of PC and transfer of arachidonic acid to another lysophospholipid, commonly lysophosphatidylethanolamine, generating lysophosphatidylcholine and PE. The intermediate product in this reaction sequence is arachidonyl-CoA, whereas cleavage of the arachidonic acid from PC by PLA<sub>2</sub> would generate free arachidonic acid. The addition of excess cold arachidonic acid does not inhibit the transfer of arachidonyl moieties from PC to PE, providing further evidence that the transfer does not reflect reacylation of free arachidonic acid released by the action of PLA<sub>2</sub> (Trotter *et al.*, 1982). The presence of the acyltransferase operating in reverse can lead to a direct ATP-independent formation of acyl-CoA ester. Since ACLS exhibits a high preference for arachidonyl-CoA, the reverse reaction could provide a specific cleavage of arachidonate from glycerophospholipids and may contribute to the redistribution of arachidonate over lipid classes (Nijssen, Oosting, Nykamp and van den Bosch, 1986). This would explain why, when labelling guinea-pig endometrium with [<sup>3</sup>H]-AA, more of the [<sup>3</sup>H]-AA goes into PC rather than into PE, yet most of the endogenous AA is found in PE (Leaver, Ning and Poyser, 1983).

#### **1:24:4 Transacylase reactions**

Arachidonic acid remodelling can also take place via two arachidonyl-specific transacylases, one being CoA-dependent (Kramer *et al.*, 1984) and the other being CoA-independent (Kramer and Deykin, 1983). In the latter study the transacylase enzyme present in human platelets demonstrated a high specificity towards the acyl donor PC transferring fatty acids in the order of preference of arachidonic acid > eicosatrienoic acid > oleic acid and preferentially acylating lysoplasmenylethanolamine rather than lysophosphatidylethanolamine. This is in contrast to platelet

acyltransferase which acylates ethanolamine lysophosphates with free arachidonic acid in the order of lysophosphatidylethanolamine > lysoplasménylethanolamine.

#### **1:24:5 Arachidonic acid turnover and pregnancy**

It has been previously discussed (section 1:14) that in the guinea-pig, the presence of conceptuses inhibits uterine  $\text{PGF}_{2\alpha}$  synthesis (Blatchley *et al.*, 1975b) by preventing the stimulatory influence of oestradiol on endometrial  $\text{PGF}_{2\alpha}$  synthesis (Poyser, 1984a). The mechanism of this inhibition is unclear, except that some anti-luteolytic factor, most probably a protein, is produced. In other species, such as the sheep and cow, the conceptus secretes a protein (ovine or bovine trophoblast protein-1) which prevents the stimulation of  $\text{PGF}_{2\alpha}$  output from the uterus (Anthony, Helmer, Sharif, Roberts, Hansen, Thatcher and Bazer, 1988). The conceptus proteins belong to a new class of interferons, namely interferon- $\tau$  (see Poyser, 1995).

Human uterine tissue, including decidua and fetal membranes, has been shown to be greatly enriched with arachidonic acid in the *sn*-2 position of phospholipids (Okita, Johnston and MacDonald, 1983). Although the stimulus for this selective uptake of arachidonic acid into uterine phospholipids is unclear, evidence from experiments with perfused human endometrium demonstrated a marked enhanced capacity for release of  $\text{PGF}_{2\alpha}$  in the late luteal phase (Skinner, Liggins, Wilson and Neale, 1984). This would suggest that such a specific uptake may be due to the action of progesterone on an oestradiol-primed tissue. The clinical observation of absence of dysmenorrhoea in anovulatory menstrual cycles is in keeping with this hypothesis. More recently, other workers isolated a 58 kd protein from amniotic fluid that specifically inhibits arachidonic acid release from dispersed, perfused decidual cells (Wilson, Liggins and Joe, 1989). The protein isolated from women in labour was found to be inactive and in smaller quantities than that obtained before the onset of labour. It was therefore proposed that this protein secreted by the chorion throughout pregnancy and, by diffusing into nearby tissues, inhibits PG synthesis and contributes to the maintenance of pregnancy. At term, the protein is inactivated and inhibition of PG synthesis ceases, facilitating the onset of labour.

In a study measuring the incorporation of [ $^{14}\text{C}$ ]-arachidonic acid into ovine conceptus and endometrial lipids, triacylglycerols were shown to be an important reservoir for both conceptual and endometrial arachidonic acid (Lewis, Wood and Caldwell, 1992). Although phospholipids are regarded to be the primary source of arachidonic acid for PG synthesis, arachidonic acid may very well be transferred from neutral lipids to phospholipids, liberated from the phospholipids and then used for PG synthesis (Lewis and Waterman, 1985). Indeed, arachidonic acid, as suggested from studies on human endometrium, may be cycled through several lipid stores prior to its



use in PG synthesis (Bonney, Samih and Franks, 1990). Studies with the guinea-pig uterus have also indicated that arachidonic acid can be transferred from "storage reservoirs" to reservoirs that provide the immediate substrate for PG synthesis (Poyser, 1991).

Conceptuses from several species synthesize PGs *in vitro* (Lewis, 1989). If conceptuses produce PGs, they must have the ability to manage the flux of esterified arachidonic acid from lipid stores to free arachidonic acid available for PG synthesis. If so, this may provide a mechanism by which the conceptus prevents the release of uterine PGF<sub>2α</sub> during early pregnancy, thus preventing luteal regression.

PGs produced by the uterus and conceptus during pregnancy have been proposed to have a variety of roles including, intrauterine migration of embryos, blastocyst hatching from the zona pellucida, ion transport across the trophoctoderm, fluid accumulation in the blastocoele, increased endometrial capillary permeability, blastocystic glucose metabolism, regulation of blood flow, immunosuppression and induction of labor (Lewis, 1989; Kelly, 1994). Therefore, the rate of arachidonic acid turnover during pregnancy may be an important factor in the control of PG synthesis by both the endometrium and the conceptus.

### **1:25 Summary and aims**

The processes controlling arachidonic acid uptake into and between phospholipids are of major importance in ensuring a continuous supply of arachidonic acid for endometrial PG synthesis. An increase in the rate of arachidonic acid uptake may be beneficial in providing a more rapid turnover of arachidonic acid for PG synthesis. However, such an increase may also limit the amount of PGs synthesized since, following its release by phospholipase, the amount of free arachidonic acid available will be reduced at a faster rate if the uptake processes for arachidonic acid are stimulated. This may be of consequence during early to mid-pregnancy when PG output from the uterus must be kept low. Likewise, reduced uptake of arachidonic acid may lead to an abnormal or overproduction of uterine PGs, which may explain certain menstrual dysfunctions such as dysmenorrhoea and menorrhagia. Indeed, inhibition of acyl-CoA synthetase and acyl-CoA:lysophospholipid acyltransferase in macrophages and lung fibroblasts resulted in increased concentrations of free arachidonic acid and PGs synthesized and released (Kroener *et al.*, 1981; Hunter *et al.*, 1984; Goppelt-Streube *et al.*, 1986).

Consequently, the aim of this study has been to investigate the presence of the enzymes which regulate arachidonic acid uptake into phospholipids in the control of PG synthesis in the endometrium. The presence of endometrial acyl-CoA synthetase

(ACS), acyl-CoA:lysophospholipid acyltransferase (ACLS) and, for comparison, phospholipase (PL) A<sub>2</sub> have therefore been investigated in guinea-pig uterus and the influences of stage of oestrous cycle, ovarian steroid hormones and pregnancy on the presence of the enzymes has been studied. The presence of the enzymes have also been investigated in fetal tissues at different stages of pregnancy, and the effects of inhibition of ACS, ACLS and PLA<sub>2</sub> activities on endometrial and fetal tissue PG synthesis have also been studied.



## CHAPTER 2 : MATERIALS AND METHODS

### Materials and General Methods

#### 2:1 Introduction

Many of the methods carried out in this thesis have been used in several different experiments, therefore the general details of these procedures are given in this chapter.

#### 2:2 Materials

##### Chemicals

Adenosine 5'-triphosphate  
Amphotericin B (Fungizone TM)  
Antioxidant (butylated hydroxytoluene)  
Arachidonic acid  
Aristolochic acid  
Bovine serum albumin (BSA)  
Calcium chloride  
Calcium ionophore (A23187)  
Coenzyme A (lithium salt, from yeast)  
D-Glucose  
Emulsifier Safe scintillant  
Gelatine  
Glutamine  
Glycerol  
Hydrochloric acid (HCl)  
Isopropanol (anhydrous)  
Kanamycin  
L-leucine

##### Source

Sigma Chemical Co., Poole, U.K.  
Flow Laboratories, Irvine, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
B.D.H. Chemicals Ltd., U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
B.D.H. Chemicals Ltd., U.K.  
Packard Instrument B.V. - U.S.A.  
B.D.H. Chemicals Ltd., U.K.  
Flow Laboratories, Irvine, U.K.  
Sigma Chemical Co., Poole, U.K.  
B.D.H. Chemicals Ltd., U.K.  
Sigma Chemical Co., Poole, U.K.  
Flow Laboratories, Irvine, U.K.  
Sigma Chemical Co., Poole, U.K.

##### Chemicals

L- $\alpha$ -phosphatidylcholine, $\beta$ -arachidonyl  
- $\gamma$ -staeroyl  
L- $\alpha$ -lysophosphatidylcholine, palmitoyl  
Magnesium Sulphate  
Medium 199 (plus Earles salts)  
Mercaptoethanol

##### Source

Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
B.D.H. Chemicals Ltd., U.K.  
Flow Laboratories, Irvine, U.K.  
B.D.H. Chemicals Ltd., U.K.

**Chemicals**

Neomycin sulphate  
Oestradiol benzoate  
Oleic acid (sodium salt)  
Palmitic acid  
PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>  
p-hydroxymercuribenzoic acid  
Peanut oil  
Phospholipase A<sub>2</sub> (from *Naja naja* venom)  
Potassium chloride  
Potassium dihydrogen orthophosphate  
PPO (2,5-diphenyloxazole)  
Progesterone  
Silica gel (100-200 mesh)  
Silica gel (150A)-plates for T.L.C.  
Sodium azide  
Sodium chloride  
Sodium dihydrogen orthophosphate  
Sodium hydrogen carbonate  
Sodium hydroxide  
Sulphuric acid  
Thimerosal (merthiolate)  
TMB-8 (3,4,5-trimethylbenzoic acid, 8-(dimethylamino)octyl ester hydrochloride)  
Tris (hydroxymethyl)methylamine (Tris)  
Quinacrine dihydrochloride

**Source**

Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
B.D.H. Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
Sigma Chemical Co., Poole, U.K.  
Koch-Light Laboratories Ltd., U.K.  
Whatman International, U.K.  
Hopkin and Williams Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.  
Sigma Chemical Co., Poole, U.K.

**Solvents**

Acetic acid  
Acetone  
Acetonitrile  
Ammonia solution  
Chloroform  
1,4-Dioxan  
2-Ethoxyethanol  
Ethyl acetate  
Heptane

**Source**

B.D.H. Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
Rathburn Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
Rathburn Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
Rathburn Chemicals Ltd., U.K.

**Solvents**

Hexane  
Methanol  
Pyridine  
Toluene

**Source**

Rathburn Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.  
Rathburn Chemicals Ltd., U.K.  
B.D.H. Chemicals Ltd., U.K.

**Radioactive compounds**

[5,6,8,9,11,12,14,15(n)- $^3\text{H}$ ] prostaglandin  $\text{F}_{2\alpha}$  (specific activity 179 Ci/mmol).  
[5,6,8,11,12,14,15(n)- $^3\text{H}$ ] prostaglandin  $\text{E}_2$  (specific activity 185-200 Ci/mmol).  
6-keto [5,8,9,11,12,14,15(n)- $^3\text{H}$ ] prostaglandin  $\text{F}_{1\alpha}$  (specific activity 151-170 Ci/mmol).  
L-3-phosphatidylcholine, 1-stearoyl-2-[1- $^{14}\text{C}$ ]arachidonyl (toluene:ethanol 1:1 v/v) (specific activity 56 mCi/mmol)  
L-lyso-3-phosphatidylcholine, 1-[1- $^{14}\text{C}$ ]palmitoyl (toluene:ethanol 1:1 v/v) (specific activity 56 mCi/mmol).  
[9,10(n)- $^3\text{H}$ ]Oleic acid (specific activity 10Ci/mmol).  
[5, 6, 8, 9, 11, 12, 14, 15- $^3\text{H}$ ]-arachidonic acid (specific activity 120-130 Ci/mmol).  
All radioactive compounds were supplied by Amersham International Ltd., U.K.

All the [ $^3\text{H}$ ] PG stock solutions were diluted to 5  $\mu\text{Ci/ml}$  and stored at  $-20^\circ\text{C}$  prior to use for radioimmunoassay. [ $^3\text{H}$ ]- $\text{PGF}_{2\alpha}$  and [ $^3\text{H}$ ]- $\text{PGE}_2$  were diluted in methanol and [ $^3\text{H}$ ]-6-keto- $\text{PGF}_{1\alpha}$  was diluted in acetonitrile:water (9:1).

**Antibodies**

Normal rabbit serum (NRS)  
Donkey anti-rabbit serum (DARS)

**Source**

Scottish Antibody Production Unit,  
Scotland

$\text{PGF}_{2\alpha}$  antiserum  
 $\text{PGE}_2$  antiserum  
6-keto- $\text{PGF}_{1\alpha}$  antiserum

All raised in the Department of  
Pharmacology,  
University of Edinburgh

**Gases**

95 % air and 5 %  $\text{CO}_2$   
95 %  $\text{O}_2$  and  $\text{CO}_2$

All gases were supplied by British Oxygen Co. Ltd., Guildford, U.K.

## Other Materials

### Material

### Source

Lens cleaning tissue

Whatman International, U.K.

Petri dishes (vented, 5cm diameter)

Sterilin Ltd., Teddington, U.K.

Teflon wool

Alltech Associates/Applied Science, U.K.

## 2:3 Composition of solutions

### 2:3:1 Krebs solution

Krebs solution was used during superfusion experiments.

Sodium chloride	34.5 g
Sodium hydrogen carbonate	10.5 g
D-glucose	10.5 g
Potassium chloride (10 % w/v)	17.7 ml
Magnesium sulphate (10 % w/v)	14.5 ml
Potassium dihydrogen orthophosphate (10 % w/v)	8.0 ml
Calcium chloride (1M)	12.6 ml

Made up to 5 l with distilled water and stored at 4°C. The calcium chloride was added at the end to avoid precipitation.

### 2:3:2 Culture medium

Medium 199 was developed by Morgan, Morton and Parker (1950) as a synthetic medium for the nutrition of animal cells in tissue culture. The medium contains amino acids, vitamins, nucleic acids and necessary growth factors supplemented with Earles' salts. Earles' salts are a synthetic mixture of inorganic salts and provide a "physiological" or balanced salt solution by maintaining pH (sodium bicarbonate), osmotic pressure and providing energy in the form of glucose (Table 1).

Glutamine, an essential component for maintenance of tissue in culture (Fischer, Astrup, Ehrensvar and Oehlenschlager, 1948) is not stable above -10°C. Therefore, glutamine was stored at -20°C and added to the culture medium as required. An antibiotic and fungicide were also added to prevent bacterial and fungal growth.

Medium 199 (plus Earles' salts)	500 ml
Glutamine (200 mM)	4 ml
Amphotericin B (Fungizone <sup>TM</sup> ) (250 µg/ml)	3 ml
Kanamycin (5 mg/ml)	3 ml

The medium was dispensed into 25 ml glass bottles and stored at -20°C until required.

**Table 1. Composition of Medium 199 (modified) with Earle's salts and 2.2g/l sodium**

bicarbonate without glutamine			
Ingredient	mg/litre	Ingredient	mg/litre
L-alanine	25.00	Nicotinic acid	0.025
L-arginine HCl	70.00	Nicotinamide	0.025
L-aspartic acid	30.00	p-aminobenzoic acid	0.05
L-cysteine HCl	.0987	Pyridoxal HCl	0.025
L-cysteine disodium	23.66	Pyridoxine HCl	0.025
salt		Riboflavin	0.01
L-glutamic acid	66.82	Thiamine HCl	0.01
L-glutamine	100.0	DL- $\alpha$ tocopherol	0.01
Glutathione	0.05	phosphate disodium salt	
Glycine	50.0	Vitamin A acetate	0.1147
L-histidine HCl	21.88	CaCl <sub>2</sub> .2H <sub>2</sub> O	264.9
H <sub>2</sub> O		Fe(NO <sub>3</sub> ) <sub>3</sub> .9H <sub>2</sub> O	0.10
L-hydroxyproline	10.0	KCl	400.0
L-isoleucine	20.0	MgSO <sub>4</sub> .7H <sub>2</sub> O	200.0
L-leucine	60.0	NaCl	6800
L-lysine HCl	70.0	NaHCO <sub>3</sub>	2200
L-methionine	15.0	NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	158.3
L-phenylalanine	25.0	Adenine sulphate 10.0	
L-proline	40.0	5'AMP	0.20
L-serine	25.0	Cholesterol	0.20
L-threonine	30.0	2-deoxyribose	0.50
L-tryptophan	10.0	D-glucose	1000
L-tyrosine disodium	49.72	Guanine HCL	0.30
salt		Hypoxanthine	0.30
L-valine	25.0	D-ribose	0.50
L-ascorbic acid	0.05	Sodium acetate	36.71
Biotin	0.01	Phenol red sodium salt	17.00
Calciferol	0.10	Thymine	0.30
D-calcium-	0.01	Tween 80	5.00
pantothenate		Uracil	0.30
Choline chloride	0.50	Xanthine	0.30
Folic acid	0.01	Menapthothone sodium	0.019
i-inositol	0.05	bisulphate 3H <sub>2</sub> O	

i. Solution for PGF<sub>2α</sub> radioimmunoassay ("PGF<sub>2α</sub> diluent")

NaN<sub>3</sub>                    0.5 g

ii. Solution for 6-keto-PGF<sub>1α</sub> radioimmunoassay ("6-keto-PGF<sub>1α</sub> diluent")

### iii Solution for PGE<sub>2</sub> radioimmunoassay (" PGE<sub>2</sub> diluent ")

Sodium dihydrogen orthophosphate (1M) 56.0 ml

iv. Scintillation fluid for prostaglandin radioimmunoassays

Toluene	1500 ml
---------	---------

## 75



## **2:5 Radioimmunoassay (RIA) technique**

### **2:5:1 Introduction**

Prostanoic acid derivatives such as prostaglandins, prostacyclin and their derivatives are generally present in biological solutions at very low concentrations ( $10^{-9}$ - $10^{-11}$  M). Therefore assays with high sensitivity are required for their measurement. One such method that is widely used is that of radioimmunoassay (RIA). This is a technique developed during the 1960's which makes use of an antibody method to measure the concentration of an unknown unlabelled antigen. Specific antigen-antibody reactions are combined with the sensitivity of radioisotopic detection. The concentration of the unknown unlabelled antigen is found by comparing its inhibitory effect on the binding of radioactively labelled antigen (tracer) to a specific antibody with the inhibitory effect of known standards. Bound and unbound antigen are separated by precipitating the antigen-antibody complexes with antiserum raised against the antibody or by some other method.

### **2:5:2 Methods of radioimmunoassay**

#### i. Preparation of standards

Assay tubes (3 ml plastic insert scintillation vials) numbered 1-38 for the  $\text{PGF}_{2\alpha}$  assay and 1-35 for the  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  assays contained a set of standard solutions. Tubes 1-4 measured non-specific binding of the radioactive tracer to sites other than the specific prostaglandin binding sites e.g. the tube itself, non-specific sites on the antiserum and to other compounds in the assay. For the assay to be valid, this non-specific binding should be  $< 10\%$ . The "counting standards", tubes 5-8, only contained radioactive tracer which when averaged gave an estimate of the number of radioactive counts within each tube.

Tubes 9-35 ( $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ ) or 9-38 ( $\text{PGF}_{2\alpha}$ ) consisted of 9 or 10 standard concentrations, respectively, (Table 2) and each standard solution was dispensed in triplicate. From the results obtained with these standard solutions, a standard curve for each assay was constructed. The standard PG concentrations were made up from stock PG solutions stored in methanol at  $-20^\circ\text{C}$  at a concentration of  $1\text{ }\mu\text{g/ml}$ .  $200\text{ }\mu\text{l}$  of stock PG solution was removed and placed in a 30 ml glass vial. The methanol was blown off under a stream of air, after which the PG was resuspended in 20 ml of the appropriate diluent to give a  $10\text{ ng/ml}$  solution.  $500\text{ }\mu\text{l}$  of this solution was placed into tubes 1-4 of the assay to measure the non-specific binding (i.e. the concentration of unlabelled PG is high enough to prevent labelled PG binding to any specific sites on the antibody, and therefore any binding is non-specific). The next PG concentration was made up by taking  $10.24\text{ ml}$  of the  $10\text{ ng/ml}$  solution and adding  $9.76\text{ ml}$  of diluent. This gave a PG solution of  $5.12\text{ ng/ml}$  from which the other

**Table 2. Concentrations and volumes of standard solutions of prostaglandin (PG) F<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> used to construct each radioimmunoassay standard curve**

Tube No.	<u>Concentration of standard solution (ng/ml)</u>		Volume (μl)
	PGF <sub>2α</sub>	PGE <sub>2</sub> / 6-keto-PGF <sub>1α</sub>	
1-4	10.0	10.0	500
5-8	0.00	0.00	0.00
9-11	0.005	0.02	500
12-14	0.01	0.04	500
15-17	0.02	0.08	500
18-20	0.04	0.16	500
21-23	0.08	0.32	500
24-26	0.16	0.64	500
27-29	0.32	1.28	500
30-32	0.64	2.56	500
33-35	1.28	5.12	500
36-38	2.56	-	500

standards were made by a series of 2-fold dilutions down to 0.005 ng/ml (for PGF<sub>2α</sub>) and 0.02 ng/ml (for PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>). The 5.12 ng/ml concentration was not used for constructing the PGF<sub>2α</sub> standard curve as a maximum of 10 standards could be used by the Spline Curve-fit programme on the IBM-personal computer linked to the scintillation counter. The standards were stored at -20°C. The last four tubes of the assay contained 500 µl of diluent in duplicate (zero standards) and 500 µl of the 0.32 ng/ml standard solution in duplicate (for calculation of inter-assay coefficients of variation).

#### ii. Preparation of radioactive tracers

The amount of [<sup>3</sup>H]-PG in a 50 µl aliquot used in each assay was calculated to give between 15,000-20,000 counts per tube when counted in a scintillation counter for 4 min (PGF<sub>2α</sub> and 6-keto-PGF<sub>1α</sub>) or for 10 min (PGE<sub>2</sub>). Prior to use, the required volume was dispensed into a glass vial and the solvent blown off under a stream of air, and [<sup>3</sup>H]-PGF<sub>2α</sub> was diluted to 0.125-0.17 µCi/ml, [<sup>3</sup>H]-PGE<sub>2</sub> was diluted to 0.05-0.0625 µCi/ml, and [<sup>3</sup>H]-6-keto-PGF<sub>1α</sub> to 0.17-0.2 µCi/ml in the appropriate diluent.

#### iii. Preparation of antibodies

The dilution of antiserum used in the radioimmunoassay was that which bound 60 % of the [<sup>3</sup>H]-PG in the absence of non-radioactive standard PG (zero standard). Antibodies were stored at 100-fold dilutions in the appropriate diluent at -20°C, and diluted to the required dilution (1/1200 for PGF<sub>2α</sub> and 6-keto-PGF<sub>1α</sub> and 1/150 for PGE<sub>2</sub>) prior to use. Donkey anti-rabbit serum (DARS) and normal rabbit serum (NRS) were stored at -20°C and diluted immediately prior to use. DARS was used 10-fold diluted in all PG assays, and NRS was used at a 100-fold dilution. The NRS was added to ensure that, at high dilutions of the antiserum, there was sufficient gamma-globulin present for adequate precipitation of the bound prostaglandins. However, no NRS was required in PGE<sub>2</sub> assays since a low dilution of PGE<sub>2</sub> antiserum had been used.

#### iv. Cross-reactivities of antisera

**PGF<sub>2α</sub>** - Antisera to PGF<sub>2α</sub> had been raised in rabbits by immunizing with PGF<sub>2α</sub> conjugated to bovine serum albumin (BSA) (Dighe, Emslie, Henderson, Rutherford and Simon, 1975). The % cross-reactivity of the antiserum was determined by finding the concentration of PG or PG metabolite which resulted in 30 % binding with the zero standard (i.e. 50 % drop from 60 % binding for the zero standard), and comparing it to the concentration of PGF<sub>2α</sub> which also produced 30 % binding with the zero standard. The % cross-reactivity was calculated using the following formula:

Concentration of PGF<sub>2α</sub> resulting in 30 % binding

x 100

Concentration of PG or PG metabolite resulting in 30 % binding

These cross-reactivities previously determined by Dighe *et al.* (1975), Poyser and Scott (1980) and Lytton and Poyser, (1982a) are shown in Table 3. The only significant cross-reactivity with PGF<sub>2α</sub> antibody used was with PGF<sub>1α</sub> (28 %). However, work by Poyser (1983a) analysing prostaglandins produced by the guinea-pig uterus by gas chromatography-mass spectrometry (GC-MS) has shown that PGF<sub>1α</sub> production is only 1-2 % of total PGF production. Therefore the PGF<sub>2α</sub> antiserum will measure predominantly PGF<sub>2α</sub> from guinea-pig uterus.

**PGE<sub>2</sub>** - Antisera to PGE<sub>2</sub> had been raised in rabbits immunized with PGE<sub>2</sub> conjugated to thyroglobulin using the method of Dighe, Smith, Ungar and Whelpdale (1978). The cross-reactivities as determined previously by Poyser and Scott (1980) and Lytton and Poyser (1982a) are shown in Table 4. PGE<sub>2</sub> antiserum was found to have significant cross-reactivity with PGE<sub>1</sub>, PGA<sub>2</sub> and PGB<sub>2</sub>. However none of these 3 PGs have been detected in extracts from guinea-pig uterus when analyzed by gas chromatography and mass spectrometry (Poyser, 1983a). Therefore the PGE<sub>2</sub> antiserum is specifically measuring PGE<sub>2</sub> in the guinea-pig uterus.

**6-keto-PGF<sub>1α</sub>** - Antisera to 6-keto-PGF<sub>1α</sub> had been raised in this department in rabbits immunized with 6-keto-PGF<sub>1α</sub> conjugated to thyroglobulin (Dighe, Jones and Poyser, 1978). The cross-reactivities determined previously (Poyser and Scott, 1980) are shown in Table 5. PGE<sub>2</sub>, PGE<sub>1</sub> and PGF<sub>1α</sub> were all found to cross-react with 6-keto-PGF<sub>1α</sub> antiserum. However, the levels were sufficiently low (< 5 %) to enable use of the antiserum for the specific measurement of 6-keto-PGF<sub>1α</sub>.

#### v. Assay procedure

All samples to be assayed for prostaglandin content were dispensed in duplicate. The appropriate volume was taken so that the amount of prostaglandin being measured was within the detection limits of the assay (ideally binding lay on the steepest part of the standard curve).

Ethyl acetate extracts of prostaglandins, which had been obtained from superfusion experiments, were taken to dryness at 45°C in a heating block under a stream of air. 500 µl of diluent were added to the dried residue samples.

Samples from tissue culture experiments were allowed to thaw before being dispensed out in duplicate (diluted if necessary) and 500 µl of diluent were added to

**Table 3. Cross-reactivities of prostaglandin F<sub>2α</sub> from rabbit 6, 6th bleed with various prostaglandins and their metabolites at 30% binding with tracer**

<b>Prostanoid</b>	<b>% Cross-reactivity</b>
PGF <sub>2α</sub>	100
PGF <sub>1α</sub>	28
PGE <sub>2</sub>	0.17
PGE <sub>1</sub>	0.54
PGA <sub>2</sub>	0.04
PGB <sub>2</sub>	0.01
PGD <sub>2</sub>	0.38
15-keto-PGF <sub>2α</sub>	0.45
13,14-dihydro-15-keto-PGF <sub>2α</sub>	0.21
15-keto-PGE <sub>2</sub>	0.01
13,14-dihydro-15-keto-PGE <sub>2</sub>	0.01
6-keto-PGF <sub>1α</sub>	0.47
TXB <sub>2</sub>	0.58

**Table 4. Cross-reactivities of prostaglandin E<sub>2</sub> antiserum from rabbit 5, 6th bleed with various prostaglandins and their metabolites at 50% binding with tracer**

<b>Prostanoid</b>	<b>% cross-reactivity</b>
PGE <sub>2</sub>	100
PGE <sub>1</sub>	66
PGA <sub>2</sub>	26
PGB <sub>2</sub>	12
PGF <sub>2α</sub>	1.5
PGD <sub>2</sub>	0.4
15-keto-PGF <sub>2α</sub>	0.1
13,14-dihydro-15-keto-PGF <sub>2α</sub>	< 0.1
15-keto-PGE <sub>2</sub>	0.2
13,14-dihydro-15-keto-PGE <sub>2</sub>	0.4
TXB <sub>2</sub>	0.2



**Table 5. Cross-reactivities of 6-keto-prostaglandin F<sub>1α</sub> antiserum from rabbit NP1, 6th bleed with various prostaglandins and their metabolites at 30% binding with tracer**

<b>Prostanoid</b>	<b>% cross-reactivity</b>
6-keto-PGF <sub>1α</sub>	100
PGF <sub>1α</sub>	0.43
PGE <sub>2</sub>	4.2
PGE <sub>1</sub>	1.1
PGA <sub>2</sub>	0.07
PGB <sub>2</sub>	0.03
PGF <sub>2α</sub>	0.01
PGD <sub>2</sub>	0.01
15-keto-PGF <sub>2α</sub>	0.04
13,14-dihydro-15-keto-PGF <sub>2α</sub>	0.07
15-keto-PGE <sub>2</sub>	0.08
13,14-dihydro-15-keto-PGE <sub>2</sub>	0.09
TXB <sub>2</sub>	< 0.01

each tube. An equal amount of tissue culture medium was added to the prostaglandin standard solutions to account for any changes in binding due to the medium.

All tubes were then treated in exactly the same way except for the "counting standards" (tubes 5-8) which only contained 50  $\mu$ l of radioactive tracer. The appropriate [ $^3$ H]-PG tracer (50  $\mu$ l) and antiserum (50  $\mu$ l) were both added to each tube using a Hamilton<sup>TM</sup> glass syringe contained in an automatic dispenser. Each tube was then "whirlmixed" and left to incubate for 1 h (PGF<sub>2 $\alpha$</sub> ) or 2 h (PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub> ) at room temperature. 50  $\mu$ l of Normal rabbit serum (NRS) and 50  $\mu$ l of Donkey anti-rabbit serum (DARS) were then added to each tube (except for PGE<sub>2</sub> assay when no NRS is required). The tubes were "whirlmixed" once more and incubated overnight at 4°C.

The following morning, all the tubes (except for the counting standards) were centrifuged at 1300 x g for 30 min at 4°C. The supernatant was immediately discarded by rapid pouring off (so that the precipitate remained firmly attached to the bottom of each tube) and the tubes allowed to dry for 15 - 30 min. Scintillation fluid (2.5 ml) was added to every tube (including the counting standards), the tubes capped and "whirlmixed" and then placed into a Canberra Packard Series 4000 liquid scintillation counter in order to measure the amount of radioactivity (in counts per minute, cpm) bound in each sample. PGF<sub>2 $\alpha$</sub>  and 6-keto-PGF<sub>1 $\alpha$</sub>  assay tubes were counted for 4 min each and PGE<sub>2</sub> tubes for 10 min. The general protocol for the measurement of prostaglandin by radioimmunoassay is shown in Figure 5.

Data from the scintillation counter were passed directly to an IBM-personal computer programmed with a Packard Data Acquisition and Analysis System (PC-DAAS). The Spline Curve-fit programme was used which processes the standards according to a Modified Smooth Spline Algorithm, in which the amount of non-specific binding was subtracted from all the standards and the co-ordinates for the curve of best-fit calculated. The amount of prostaglandin present in each sample tube was determined by the computer from the standard curve. Samples that lay outside the linear part of the curve were re-assayed using a more appropriate sample volume.

#### vi. Intra-assay coefficient of variation

This coefficient determines the variation between each sample duplicate. It was calculated by the computer using the following formula:

$$\frac{\text{Standard deviation of sample duplicate}}{\text{Mean value of sample duplicate}} \times 100$$

## Figure 5. General protocol for measurement of prostaglandins by radioimmunoassay

### Superfusion samples

Dispense appropriate volume of each sample into tubes

\*\*\*\*\*

Evaporate off ethyl acetate to dryness in a stream of air at 45°C

### Culture medium samples

Thaw and dispense appropriate volume of each sample into tubes

\*\*\*\*\*

Add appropriate diluent to equal volume of the PG standards

\*\*\*\*\*

Add 50 µl of appropriate [<sup>3</sup>H]-PG to each tube

\*\*\*\*\*

Add 50 µl of appropriate PG antiserum to each tube except the counting standards

\*\*\*\*\*

“Whirlimix” and incubate tubes for 1 h (PGF<sub>2α</sub>) and 2 h (PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>) at room temperature

\*\*\*\*\*

Add 50 µl appropriate NRS (not PGE<sub>2</sub> assay) and 50 µl appropriate DARS to each tube except the counting standards

\*\*\*\*\*

Whirlimix and incubate tubes overnight at 4°C

\*\*\*\*\*

Centrifuge tubes (except counting standards) at 1300 × g for 30 min at 4°C

\*\*\*\*\*

Discard supernatant by rapid pouring off

\*\*\*\*\*

Add 2.5 ml PG scintillation fluid and cap every tube

\*\*\*\*\*

Whirlimix tubes to resuspend pellet

\*\*\*\*\*

Count each tube in a liquid scintillation counter for 4 min (PGF<sub>2α</sub> and 6-keto-PGF<sub>1α</sub>) or 10 min (PGE<sub>2</sub>)

The intra-assay coefficient of variation was calculated for every sample duplicate in each assay performed. If the coefficient was > 10 %, the sample was re-assayed.

#### vii. Inter-assay coefficient of variation

This coefficient determines the variation between assays and is calculated using the average value of the last two standard duplicates in each assay (0.32 ng/ml prostaglandin) from several assays using the following formula:

$$\frac{\text{Standard deviation of PG standard}}{\text{Mean PG standard}} \times 100$$

#### viii. Precision of PGF<sub>2α</sub> radioimmunoassay

The standard curve for the PGF<sub>2α</sub> radioimmunoassay is shown in Figure 6. The intra- and inter-assay coefficients of variation were 6.16 % and 3.98 % respectively. The lower limit of detection for the assay ranges from 25 to 40 pg. The accuracy and precision of the assay were considered satisfactory for the assay of PGF<sub>2α</sub>.

#### ix. Precision of PGE<sub>2</sub> radioimmunoassay

The standard curve for the PGE<sub>2</sub> radioimmunoassay is shown in Figure 7. The intra- and inter-assay coefficients of variation were 5.04% and 8.62 % respectively. The lower limit of detection for the assay ranges from 10 to 35 pg. The accuracy and precision of the assay were considered satisfactory for the assay of PGE<sub>2</sub>.

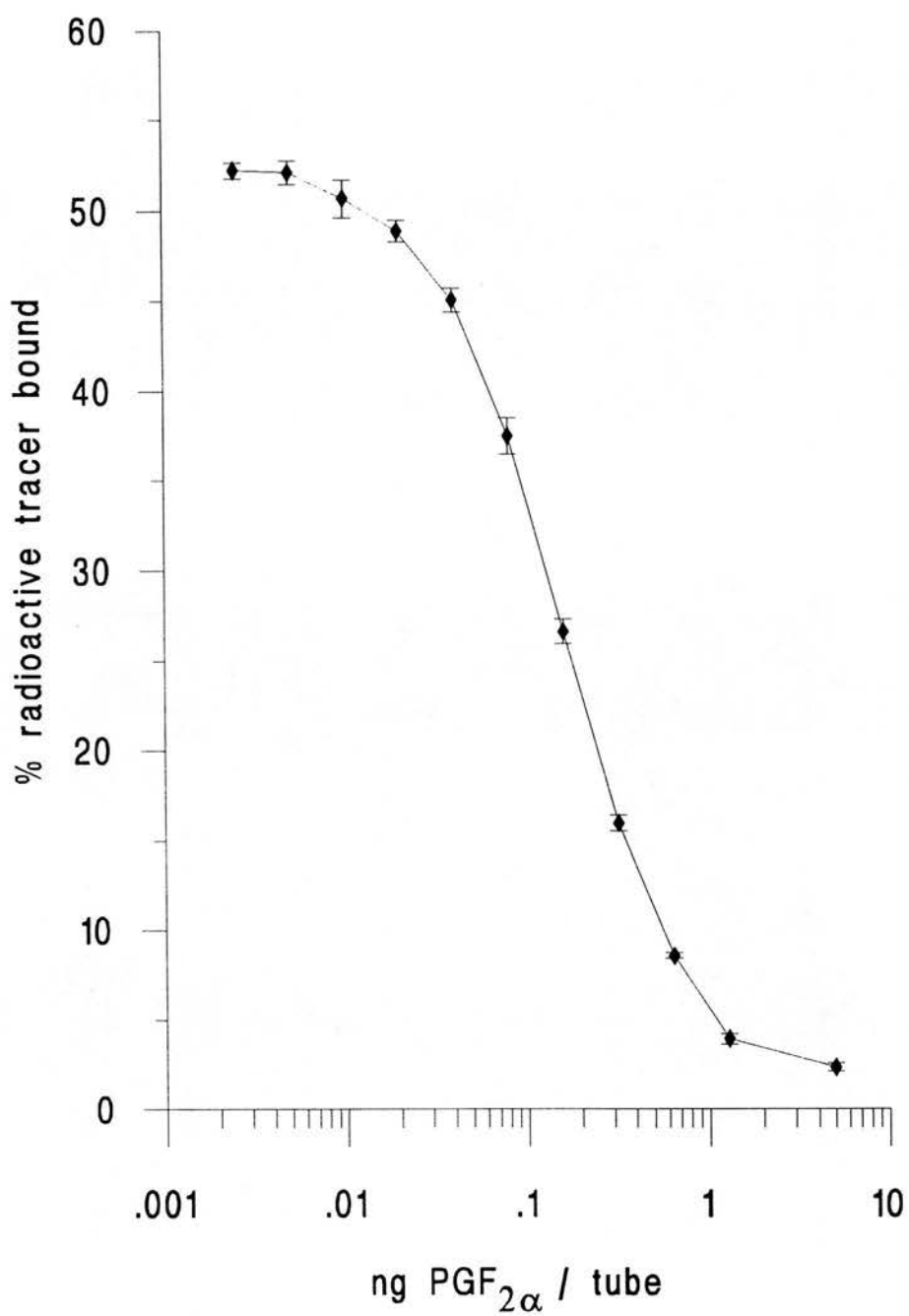
#### x. Precision of 6-keto-PGF<sub>1α</sub> radioimmunoassay

The standard curve for the 6-keto-PGF<sub>1α</sub> radioimmunoassay is shown in Figure 8. The intra- and inter-assay coefficients of variation were 6.35 % and 5.28 % respectively. The lower limit of detection for the assay ranges from 10 to 40 pg. The accuracy and precision of the assay were considered satisfactory for the assay of 6-keto-PGF<sub>1α</sub>.

## 2:6 Tissue culture technique

### 2:6:1 Introduction

Tissue was cultured by a method based on that described by Baker and Neal (1969) for ovarian tissue, and further refined for the culture of endometrial tissue by Abel and Baird (1980), Leaver and Poyser (1982), Ning, Leaver and Poyser (1983) and Leckie and Poyser (1990). Tissue was cultured for 24h. Previous histological examination (Leaver and Seawright, 1982; Ning, Leaver and Poyser, 1983; Ning and Poyser, 1984) have shown that guinea-pig endometrium remains viable during tissue



**Figure 6. Standard curve for PGF<sub>2α</sub> radioimmunoassay. Each point is the mean  $\pm$  s.e.m. (n = 5).**

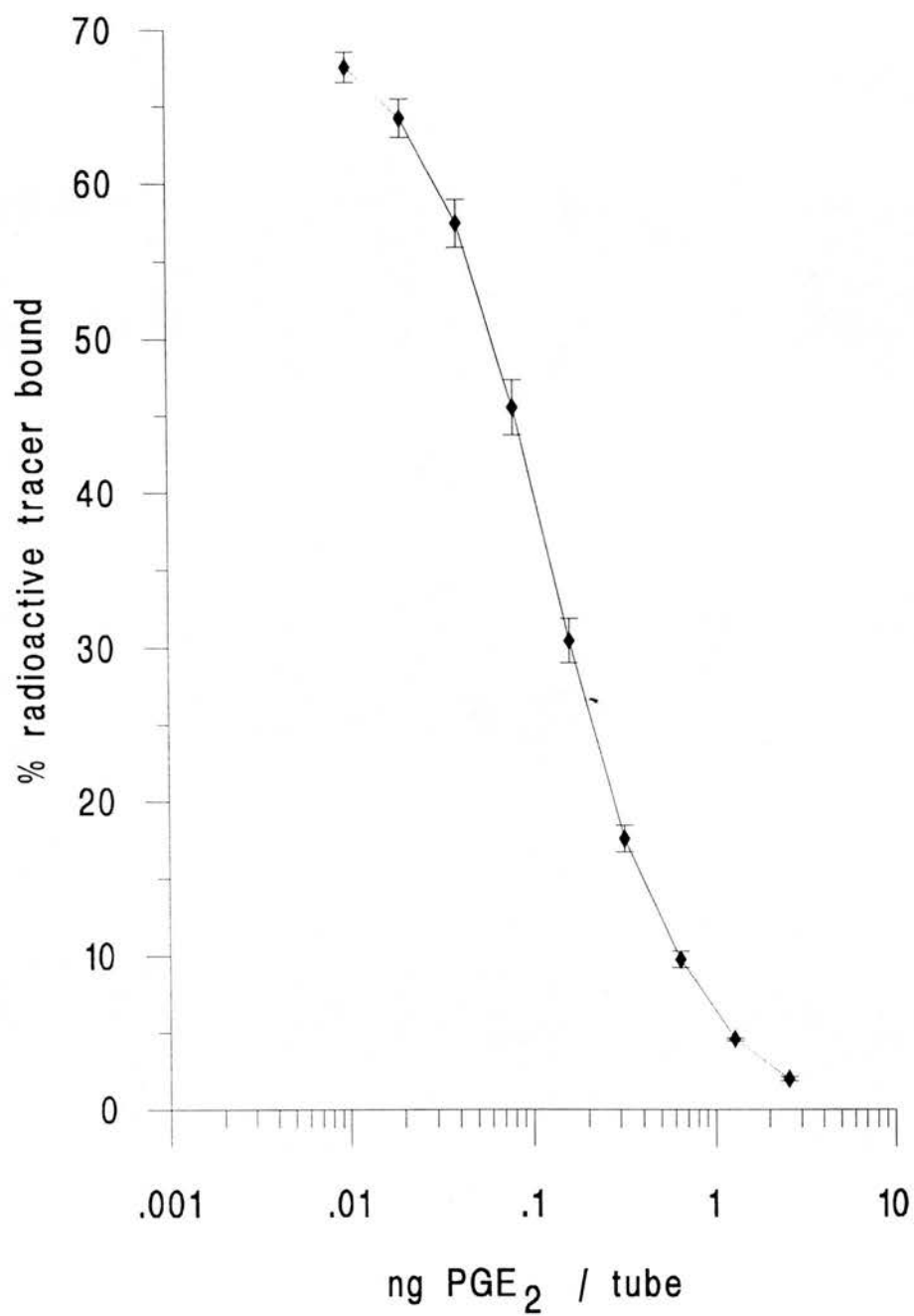
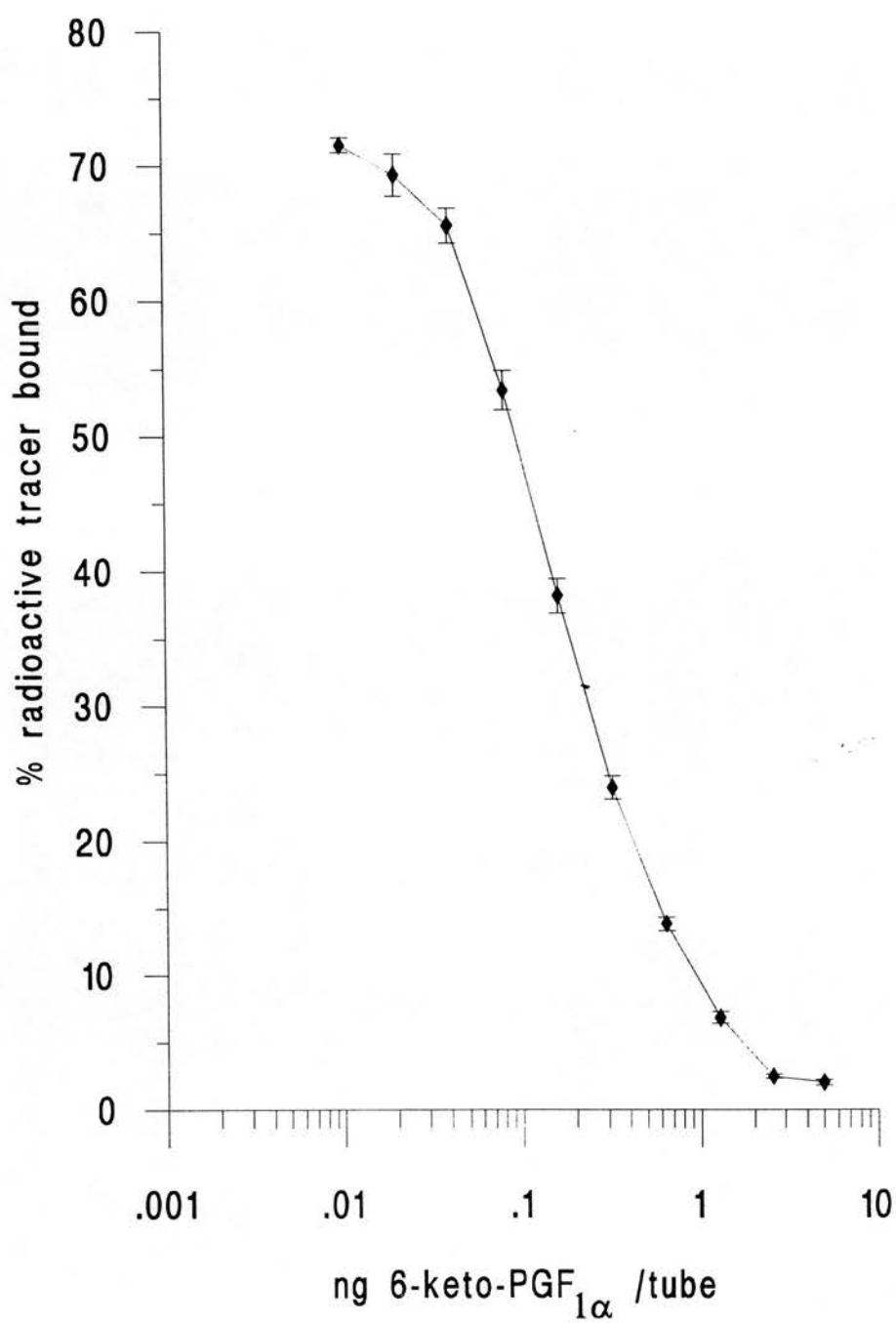


Figure 7. Standard curve for PGE<sub>2</sub> radioimmunoassay. Each point is the mean  $\pm$  s.e.m. (n = 5).





**Figure 8. Standard curve for 6-keto-PGF<sub>1α</sub> radioimmunoassay. Each point is the mean ± s.e.m. (n = 5).**

culture for periods of up to 72 h. All experiments were carried out under strict aseptic conditions within a "Microflow hood". All glass and plastic utensils were autoclaved prior to use, and sterile culture dishes (5 cm, vented) were employed. Surgical instruments were kept in ethanol, and flamed before use.

### **2:6:2 Preparation of tissue**

#### **i. Endometrium from day 7 and 15 non-pregnant guinea-pig**

The uterus was removed from day 7 or 15 guinea-pigs, the uterine horn was opened via a longitudinal incision, and the endometrium was carefully dissected from the myometrium using a pair of fine scissors. This technique produces greater than 85 % separation (Leaver and Poyser, 1981).

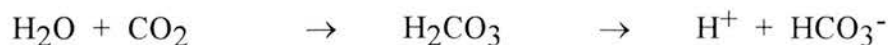
#### **ii. Endometrial, conceptual and fetal tissue from pregnant guinea-pigs**

The uterine horns were removed from guinea-pigs on days 15, 22, 29 and 36 of pregnancy and each horn was opened carefully as described above. In day 15 pregnant animals, the conceptuses were gently removed. In day 22, 29 and 36 pregnant animals, the fetuses were gently pulled away from the endometrium and separated into subplacenta, chorio-allantoic placenta, chorion and amnion tissue. Endometrial tissue from day 15, 22, 29 and 36 pregnant animals was removed as described for day 15 non-pregnant guinea-pigs.

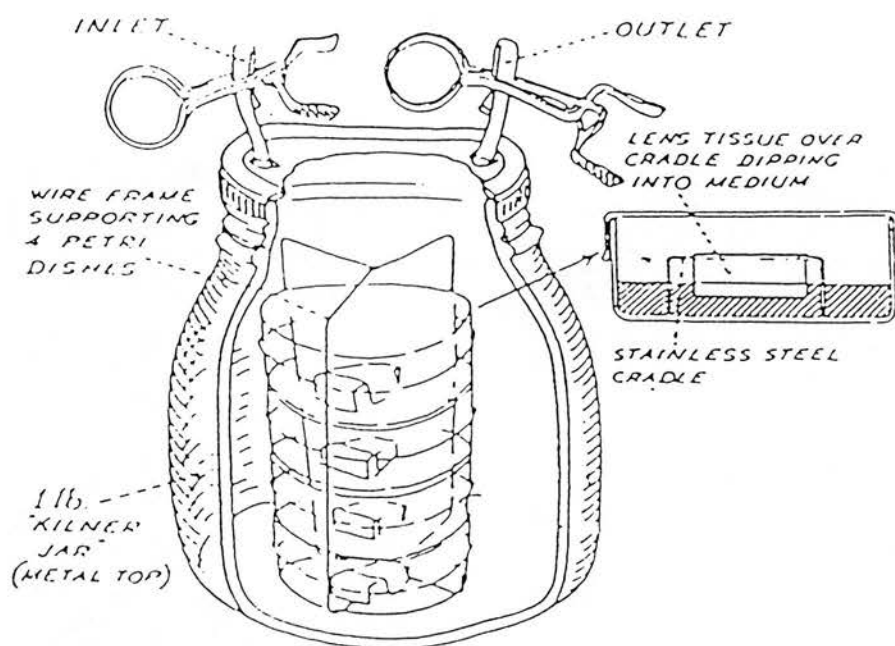
### **2:6:3 Method of tissue culture procedure**

Separated tissue (endometrial, conceptual or fetal) was cut into 1-2 mm<sup>3</sup> pieces and placed on a section of lens tissue lying across a raised stainless steel platform in a petri dish containing 4 ml culture medium plus any other necessary compounds according to the nature of the experiment, as detailed later in the results chapter. All materials used were sterile. The lens tissue had been placed in such a way that it was in contact with the supplemented culture medium. Thus the tissue was not immersed in medium, but had access to it via capillary action through the lens tissue. Approximately 20 mg of tissue were placed in each petri dish.

Petri dishes were stacked in a steel rack and placed in a modified Kilner jar (Figure 9). Each jar was pressurised to 0.7 kg/cm<sup>2</sup> with a 1:1 mixture of air/CO<sub>2</sub> (95%:5%) and O<sub>2</sub>/CO<sub>2</sub> (95%:5%). A small amount of CO<sub>2</sub> is necessary to maintain the pH of the culture medium by creating a CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> situation.



The amount of CO<sub>2</sub> dissolved is directly related to the incubation temperature and the CO<sub>2</sub> will combine with the H<sub>2</sub>O to produce H<sub>2</sub>CO<sub>3</sub> which dissociates. HCO<sub>3</sub><sup>-</sup> tends to reassociate leaving the medium acid. By increasing the CO<sub>2</sub> concentration, the pH



**Figure 9. The above diagram represents the equipment used to culture guinea-pig endometrial and conceptual tissue.**

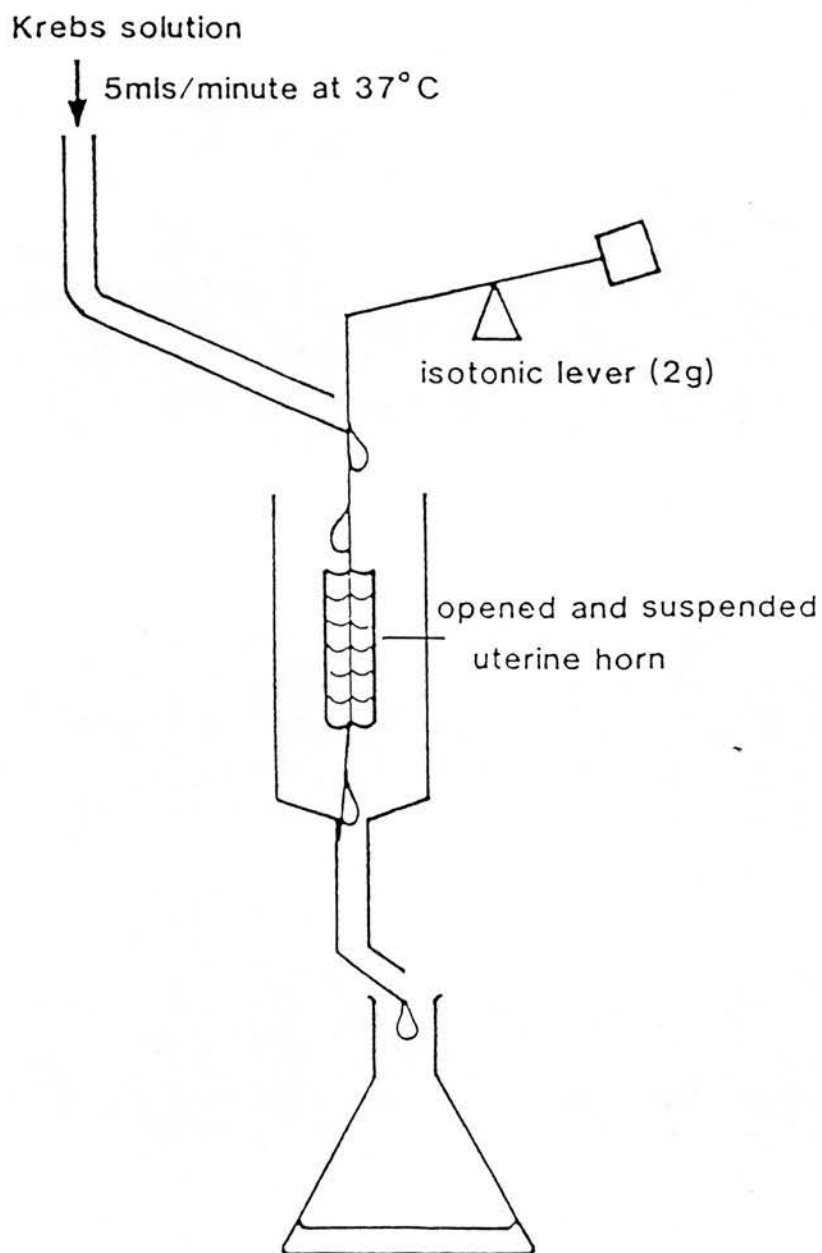
is depressed. However, the sodium bicarbonate present in the medium tends to shift the equation to the left resulting in an equilibrium of pH 7.4. If no CO<sub>2</sub> were present at 37°C, the pH would increase resulting in the sodium phenol red indicator present changing colour to purple. In such a case, the PG output by the tissue may be inhibited. During all tissue culture experiments carried out in this thesis the culture medium always remained red. The high oxygen levels in the jars overcome any diffusion problems caused by the small size of the tissue pieces.

The Kilner jars were then incubated at 37°C for the designated time of the experiment. The culture medium was collected every 6 h and replaced with prewarmed fresh medium. The jars were regassed every time a sample was taken. The experiments were all performed for a total of 24 h (i.e. medium samples taken at 6 h, 12 h, 18 h and 24 h). The samples were placed in small plastic vials and stored at -20°C until prostaglandin content was determined by radioimmunoassay. The tissue from each dish was removed and dried in an oven at 37°C for 24 h and then weighed.

## 2:7 Superfusion technique

This technique was developed by Poyser and Brydon (1983) to measure the release of prostaglandins by the superfused guinea-pig uterus. Since the guinea-pig has two uterine horns, one is used as a control measuring basal PG output while the other horn is exposed to the test condition. Both uterine horns were removed immediately after each animal was killed, and placed into Krebs solution at 4°C. Any excess vascular or fatty tissue was trimmed away, after which the horns were each blotted dry, weighed and opened via a longitudinal incision. Each horn was then suspended from an isotonic lever under a load of 2 g and superfused with Krebs solution (5 ml/min) which had been pre-aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> and heated to 37°C (Figure 10).

Each horn was superfused initially for a settling period of 60 min, and then samples collected for 10-min periods over the next 80 or 100 min (i.e. 8 or 10 samples/uterine horn respectively). After collection, the pH of each sample was lowered to 4.0 by addition of HCl. PGs were solvent extracted from the samples of superfusate with two 50 ml volumes of redistilled ethyl acetate. Both 50 ml fractions were combined and evaporated to dryness at 45°C using a Buchi rotary evaporator. Each extract was redissolved in 10 ml of redistilled ethyl acetate and stored at -20°C. During the whole superfusion period the tone of the uterine horns was noted. The amounts of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> present in the extracts were later measured by radioimmunoassay. This extraction procedure gives a high rate of recovery for PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>. The recoveries (mean ± s.e.m., n=3)



**Figure 10.** The above diagram represents the method used for superfusion of guinea-pig uterine horn *in vitro*.

of the appropriate radioactive PGs at pH 4.0 were found to be  $94 \pm 3\%$  for  $\text{PGF}_{2\alpha}$ ,  $92 \pm 5\%$  for  $\text{PGE}_2$  (Poyser and Scott, 1980) and  $82 \pm 2\%$  for 6-keto- $\text{PGF}_{1\alpha}$  (Swan and Poyser, 1983). PG concentrations measured in samples of superfusate have not been corrected for these losses during recovery.

## **2:8 Uterine homogenate incubations**

The technique of whole uterine homogenisation in order to measure prostaglandin production was first described by Poyser (1972). Specific measurement of endometrial prostaglandins were later achieved by separation of the endometrium from the myometrium (Poyser, 1983a).

The uterus was removed from day 7 guinea-pigs and placed into ice-cold Krebs solution. Each uterine horn was opened via a longitudinal incision, and the endometrium was carefully dissected from the myometrium using a pair of fine scissors. The endometrium from a single guinea-pig was then blotted dry, weighed and cut into fine pieces. It was then homogenised in 10 ml ice-cold Krebs solution (plus any specified drug additions as described in results chapter) using a Jencons ground glass homogeniser. The time interval between removal of the uterus and completion of homogenisation was never greater than 15 min. The homogenate was then oxygenated and incubated for 1 h at  $37^\circ\text{C}$  in a shaking water bath. After incubation, the pH of the incubates was lowered to pH 4.0 by addition of HCl and the prostaglandins were extracted twice with 15 ml redistilled ethyl acetate as described in section 2:7. The prostaglandin content was determined by radioimmunoassay.



## **Development and Modifications to Enzyme Assays**

### **2:9 Introduction**

The following section describes the procedures taken to develop three enzyme assays to investigate the presence of three enzymes, namely acyl-CoA synthetase (ACS), acyl-CoA:lysophospholipid acyltransferase (ACLS) and phospholipase (PL)<sub>A2</sub> in various tissues from non-pregnant and pregnant guinea-pigs. The assays were developed separately, but once all were set up and running, all three were performed simultaneously using tissue from the same animal.

### **2:10 Preparation of microsomal pellet**

#### **2:10:1 Introduction**

Preparation of a microsomal pellet was the initial step necessary for all three assays and is therefore described in this section. The procedure used was that of Downing and Poyser (1983) who observed that between 85 to 95% of PLA<sub>2</sub> activity was present in the microsomal rather than in the supernatant fraction of guinea-pig endometrial homogenates following centrifugation at 100,000 x g for 60 min. ACS, ACLS and PLA<sub>2</sub> activities have been detected in the microsomal pellet isolated from guinea-pig endometrial and fetal tissue.

#### **2:10:2 Procedure**

Female guinea-pigs were killed by stunning and rapid incision of the neck. Non-pregnant animals on days 7 and 15 of the cycle, pregnant animals on days 15, 29 and 36 of gestation, ovariectomised animals which had received steroid hormone treatment (as outlined in the results section) were used. Both uterine horns were immediately removed and placed into ice-cold Tris buffer (one uterine horn in 0.05 M, pH 8.0 for ACS and PLA<sub>2</sub> assays, and the other uterine horn in 0.05 M pH 7.5 for ACLS assay). Each uterine horn was opened longitudinally and the endometrium dissected away from the myometrium by cutting away 1-2 mm<sup>3</sup> sections of endometrium using a pair of fine scissors. In pregnant animals, the conceptuses (day 15 pregnant) or the fetal tissues (i.e. subplacenta, chorio-allantoic placenta, chorion and amnion in day 29 and 36 pregnant animals) were also removed, gently separated and each tissue type equally divided between the two buffers.

The endometrium and fetal tissues were homogenised separately in Tris buffer (0.05 M, pH 8.0 for ACS and PLA<sub>2</sub> assays, and 0.05 M pH 7.5 for ACLS assay) using a ground glass homogeniser followed by sonication to give a 25 % homogenate (250 mg in 1 ml buffer). This homogenate was then centrifuged for 15 min at 3000 x g at 4°C, after which the supernatant fluid was removed and centrifuged for 60 min at



### **2:11:2 Measurement of arachidonyl-CoA synthetase activity**

Wilson *et al.* (1982) utilized human platelet membrane, and rat liver microsomal, preparations. The microsomal pellet from guinea-pig uterine tissue used in subsequent experiments was prepared as described in section 2:10 and was resuspended in 0.25 ml 0.05 M Tris-HCl, pH 8.0. Two 10  $\mu$ l portions were removed and used for estimation of protein content by the method of Lowry, Rosebrough, Farr and Randall (1951) (section 2:15). The enzyme assay was performed in duplicate, and thus two 50  $\mu$ l portions of enzyme were taken and used as shown in Figure 11. Aliquots of 60  $\mu$ l of "reaction mix" and 40  $\mu$ l of a 0.5 mM solution of radiolabelled fatty acid in 50 mM NaHCO<sub>3</sub> were placed in an eppendorf tube kept ice-cold. The cold fatty acid used for this assay was arachidonic acid along with [<sup>3</sup>H]-arachidonic acid in order to measure specific arachidonyl-CoA synthetase activity. The reaction was initiated by addition of the enzyme to the eppendorf, and incubated at 37°C for 10 min. The reaction mixture was then immediately added to a 25 ml pear-shaped separating flask containing 2.25 ml of isopropanol:heptane:2 M sulphuric acid (40/10/1, by volume) which terminated the reaction. Heptane (1.5 ml) and 1 ml distilled water were then added and the mixture vortexed vigorously. The upper phase was withdrawn and placed into a 20 ml scintillation vial. The aqueous phase remaining in the separating flask was extracted three times with 2 ml heptane containing 4 mg/ml of carrier palmitic acid (which removes unreacted radiolabelled fatty acid). The final aqueous phase contained the soluble long chain acyl-CoA esters. Each upper phase and the final aqueous phase were also placed into 20 ml scintillation vials. Emulsifier Safe scintillant (10 ml) was added to each vial, and all the vials were vortexed vigorously. They were then allowed to equilibrate for 1 h prior to liquid scintillation counting in a Canberra Packard Tricarb 4000 series liquid scintillation analyser. The activity of acyl-CoA-synthetase was calculated as the conversion of [<sup>3</sup>H]-fatty acid to [<sup>3</sup>H]-fatty acid-CoA during a 10 min incubation period as a percentage of the total radioactivity recovered. In the methods of Wilson *et al.* (1982) only the aqueous phase (1 ml) (containing the soluble long chain acyl-CoA esters) was collected and counted in a scintillation counter. However, it was felt, that by collecting and scintillation counting all the phases, there would possibly be a more accurate detection of acyl-CoA-synthetase, since activity was calculated as a percentage of total radioactivity recovered, rather than as radioactivity recovered compared to original radioactivity in the reaction mix.

### **2:11:3 Identification of reaction product**

The radiolabelled product collected in the arachidonyl-CoA synthetase assay was shown to be arachidonyl-CoA by co-chromatography with authentic arachidonyl-

## Figure 11. General Protocol for measuring acyl-CoA synthetase activity

60  $\mu$ l "reaction mix" (i) + 40  $\mu$ l of a 0.5 mM solution of radiolabelled fatty acid in 50 mM NaHCO<sub>3</sub> (ii) + 50  $\mu$ l enzyme (iii)

Total reaction mixture : 150  $\mu$ l (i + ii + iii)

\*\*\*\*\*

Incubated for 10 min at 37 °C

\*\*\*\*\*

Add 2.25 ml isopropanol:heptane:H<sub>2</sub>SO<sub>4</sub> (40:10:1)

\*\*\*\*\*

Add 1.5 ml heptane + 1.0 ml water

Vortex vigorously

\*\*\*\*\*

Upper phase removed

\*\*\*\*\*

Aqueous phase extracted 3 times with 2 ml heptane containing 4 mg/ml palmitic acid

\*\*\*\*\*

Aqueous phase remaining removed

\*\*\*\*\*

10 ml Scintillation fluid added to each removed phase

\*\*\*\*\*

Vortexed vigorously

\*\*\*\*\*

Vials scintillation counted for 4min

CoA using the method of Mishina, Kamiryo, Tashiro and Numa (1978) in a solvent system of isopropanol:pyridine:acetic acid:water (60:15:1:25). It was found that 80-90 % of the radioactivity co-chromatographed with the authentic arachidonyl-CoA. The  $R_f$  value was found to be 0.29; these results agree with those of Wilson *et al.* (1982), and therefore this extraction method was assumed to be predominantly collecting arachidonyl-CoA.

## **2:12 Measurement of AcylCoA:lysophospholipid acyltransferase activity**

### **2:12:1 Introduction**

Arachidonyl-CoA:lysophospholipid acyltransferase activity was measured in guinea-pig microsomal pellet using a modified version of the procedures described by Severson and Fletcher (1984) and Fuse, Iwanaga and Tai (1989). The conversion of labelled lysophosphatidylcholine to phosphatidylcholine in the presence of donor arachidonyl-CoA was measured. The general procedure is shown in Figure 12.

### **2:12:2 Assay procedure**

#### i. Acyl-CoA:lysophospholipid acyltransferase assay

The acyltransferase activity was routinely determined in duplicate in eppendorf tubes in assay incubations (0.2 ml final volume) containing 250  $\mu$ M cold L- $\alpha$ -lysophosphatidylcholine, palmitoyl (LPC) with 0.1  $\mu$ Ci (1.78 nmol, approximately 200,000 dpm per assay) L-lyso-3-phosphatidylcholine-1( $1-^{14}$ C)-palmitoyl (solvent base blown off under stream of  $N_2$  and redissolved in cold LPC 0.05M Tris-HCl solution, pH 7.5), 20 nmol arachidonyl-CoA, 5 mM  $MgCl_2$  and a source of enzyme (resuspended microsomal pellet in 0.25 ml 0.05M Tris pH 7.5). Two 10  $\mu$ l volumes of resuspended microsomal pellet were taken for estimation of protein content. The reaction was initiated by the addition of the enzyme and incubated at 37°C for 10 min, after which the reaction mixture was immediately placed into a 10 ml glass centrifuge tube containing 3 ml  $CHCl_3:CH_3OH$  (2:1 v/v) with 0.05% antioxidant to terminate the reaction. 0.1 M  $CaCl_2$  (1 ml) was also added and the mixture vortexed vigorously. The aqueous and solvent phases were separated by centrifugation at 1000 x g for 5 min. The lower phase was dried under a stream of  $N_2$ , resuspended in 50  $\mu$ l (rather than 150  $\mu$ l as used by Severson and Fletcher, 1985, as this made application of the sample easier to TLC plate - smaller sample spot achieved) of  $CHCl_3:CH_3OH$  (2:1 v/v) and stored at -20°C until subjected to thin layer chromatography (TLC).

#### ii. Separation of LPC and PC fractions

Lysophosphatidylcholine and phosphatidylcholine fractions (labelled and unlabelled) were separated by thin layer chromatography on thin layer silica gel chromatographic glass plates. The lower phase collected in the assay was applied to

## Figure 12. General protocol for measuring acylCoA:lysophospholipid acyltransferase activity

130 $\mu$ l 0.05M Tris buffer pH 7.5 containing 20 nmol arachidonyl-CoA and 5 mM $MgCl_2$	+	20 $\mu$ l 250 $\mu$ M cold LPC containing 0.1 $\mu$ Ci [ $^{14}C$ ]-LPC (1.78 nmol)	+	50 $\mu$ l enzyme
----------------------------------------------------------------------------------------------------	---	--------------------------------------------------------------------------------------------	---	----------------------

Total reaction mixture 200  $\mu$ l

\*\*\*\*\*

Incubate for 10 min at 37°C

\*\*\*\*\*

3 ml  $CHCl_3:CH_3OH$  (2:1 v/v) containing 0.05% antioxidant  
and 1 ml 0.1 M  $CaCl_2$  added

\*\*\*\*\*

Vortex vigorously

\*\*\*\*\*

Phases separated by centrifugation at 1000 x g for 5 min

\*\*\*\*\*

Lower phase dried under stream of  $N_2$  gas

\*\*\*\*\*

Dried sample resuspended in 50  $\mu$ l  $CHCl_3:CH_3OH$  (2:1 v/v)

\*\*\*\*\*

Sample stored at -20°C until required for TLC



the plate using a microsyringe along with LPC (25 µg) and PC (10 µg) as carriers. LPC and PC markers were also applied on either sides of each TLC plate and the plates developed in a solvent system of  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$  (75:25:3) to a height of 15 cm. Once the samples had all reached the solvent front, the plates were allowed to dry flat. Plates were gently covered with a sheet of paper covering all sample columns except the marker columns. Marker LPC and PC zones were visualised by spraying on molybdo-phosphoric acid in 5% ethanol. Plates were dried in an oven at 37°C for 15 min, and the marker LPC and PC zones were clearly visible as light green spot where the molybdo-phosphoric acid had attached to the phospholipids.

The specified quantities of LPC and PC markers/carriers were selected after several TLC trial runs. These quantities (LPC (25 µg) and PC (10 µg)) achieved clear indication of their positions following solvent and molybo-phosphoric exposure. The solvent system of  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$  (75:25:3), used by Fuse et al., 1989, resulted in the best separation of LPC and PC zones (to a height of 15 cm, in approximately 1 h) compared to the solvent system used by Severson and Fletcher, 1985 ( $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}:\text{CH}_3\text{COOH}$  (280:120:16:8)). Therefore the  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$  (75:25:3) solvent system was selected for use in our experiments.

The silica gel zones corresponding to the LPC and PC in the samples were dampened using a "fine mist spray" of distilled water and scraped off separately into 5 ml scintillation vials containing 0.5 ml methanol (to extract the silica gel). During initial TLC trials, it was discovered that dampening of silica gel reduced loss of samples in the form of dust and made collection of gel much easier. Scintillation fluid (4 ml) was then added to each vial and the vials vortexed vigorously. Vials were left to equilibrate for 1 h prior to liquid scintillation counting to determine the amount of radioactivity present. ACLS activity was calculated as the conversion of [ $^{14}\text{C}$ ]-LPC to [ $^{14}\text{C}$ ]-PC during a 10 min incubation as a percentage of the total radioactivity recovered.

## **2:13 Measurement of phospholipase A<sub>2</sub> activity**

### **2:13:1 Introduction**

The procedure used for determination of PLA<sub>2</sub> activity was based on the method described originally by Consentino and Ellis (1981) and modified by Downing and Poyser (1983). The method was further modified for use in the experiments described in this thesis. The general procedure is shown in Figure 13.

### Figure 13. General Protocol for measuring PLA<sub>2</sub> activity

50 µl 0.1 M Tris, pH 8.0, 7 mM Ca<sup>2+</sup> + 50 µl enzyme  
containing 0.1 µCi [14C]-PC (1.78 nmol)  
and 70 µM cold PC sonicated prior to use  
to solubilise the phospholipids

Total reaction mixture 100 µl

\*\*\*\*\*

Incubate for 10 min at 37°C

\*\*\*\*\*

Add 25 µl 2 N HCl + 10 µg Arachidonic acid

\*\*\*\*\*

Incubates frozen overnight at -20°C prior  
to separation of PC and AA by column chromatography

## 2:13:2 Assay procedure

### i. Conversion of [ $^{14}\text{C}$ ]-PC to [ $^{14}\text{C}$ ]-AA

The microsomal pellet prepared was resuspended in  $\text{PLA}_2$  buffer (0.25 ml 0.1 M Tris, pH 8.0 containing 7 mM  $\text{Ca}^{2+}$ ) and kept at  $4^\circ\text{C}$ . Two 50  $\mu\text{l}$  volumes were taken for the assay along with two 10  $\mu\text{l}$  volumes for the purpose of protein concentration determination. Phosphatidylcholine with [ $^{14}\text{C}$ ]-arachidonic acid in the carbon-2 position (0.1  $\mu\text{Ci}$ ) was used as the labelled substrate. It was blown off from its solvent base and redissolved in an eppendorf tube with 50  $\mu\text{l}$  buffer containing 70  $\mu\text{M}$  unlabelled PC which had been previously sonicated to solubilise the phospholipids. The final reaction volume was 100  $\mu\text{l}$ . The reaction was initiated by addition of the enzyme to the phospholipids, and incubated for 10 min at  $37^\circ\text{C}$ . Addition of 25  $\mu\text{l}$  2 N HCl terminated the reaction and 10  $\mu\text{g}$  arachidonic acid were added to act as a carrier for the [ $^{14}\text{C}$ ]-AA released from the labelled substrate. The incubates were frozen at  $-20^\circ\text{C}$  overnight.

### ii. Separation of PC and AA by column chromatography

Glass pasteur pipettes plugged with teflon wool (one for each incubate) were packed with silica gel (100-200 mesh) suspended in hexane to a height of 3 cm and then washed through with 1 ml of Solvent 1 (hexane:1,4-dioxan:acetic acid, 85:15:1). The columns were not allowed to run dry at any point during the experiment. Solvents were applied to the columns with glass pasteur pipettes and allowed to drip through by gravity. Each incubate was then added to the top of a column, and the fatty acid ([ $^{14}\text{C}$ ]-AA) eluted into ten 3 ml plastic scintillation vials with ten 1 ml fractions of Solvent 1. The unmetabolised substrate ([ $^{14}\text{C}$ ]-PC) was then eluted and collected in the same way but using ten 1 ml fractions of Solvent 2 (chloroform:methanol:water, 65:35:4). During initial trial column chromatography runs, it was found that a greater recovery of the labelled PC and AA was achieved using 10 x 1 ml fractions of each solvent rather than just 6 fractions of Solvent 1 followed by 7 fractions of Solvent 2 as described by Downing and Poyser (1983). The solvents collected in each vial were evaporated off under a stream of air in a heating block at  $45^\circ\text{C}$ , after which 2.5 ml scintillation fluid were added and the vials vortex mixed. The tubes were counted in a Canberra Packard Tricarb 4000 series liquid scintillation analyser for 4 min. The  $\text{PLA}_2$  activity was calculated as the conversion of [ $^{14}\text{C}$ ]-PC to [ $^{14}\text{C}$ ]-AA during a 10 min incubation period calculated as a percentage of the total radioactivity recovered.

## **2:14 Control experiments**

### **2:14:1 Introduction**

A set of experiments was performed to try to identify potentially better conditions for each enzyme assay. The results obtained were observed in at least two different animals. Curves were drawn in to “best fit” manually, and extrapolated to zero.

### **2:14:2 Cold substrate concentration**

The cold substrate concentration is of great importance in each assay to ensure that the enzyme is not being limited, and that optimum enzyme activity is being measured. The cold substrate concentrations listed in the following experiments represent the actual concentration of cold substrate present in the reaction mix (not the concentration of cold stock solution added).

#### i. ACS assay

A set of control assay experiments were carried out using non-labelled arachidonic acid concentrations of 5, 25, 50, 100, 200, 300, 400, 500 and 600  $\mu\text{M}$ . The results are shown in Figure 14. A substrate concentration of 500  $\mu\text{M}$  was used in all further experiments.

#### ii. ACLS assay

A set of control assay experiments was carried out using cold lysophospholipid concentrations of 5, 25, 50, 100, 150, 200, 250, 300 and 350  $\mu\text{M}$ . The results are shown in Figure 15. A substrate concentration of 250  $\mu\text{M}$  was used in all further experiments.

#### iii. PLA<sub>2</sub> assay

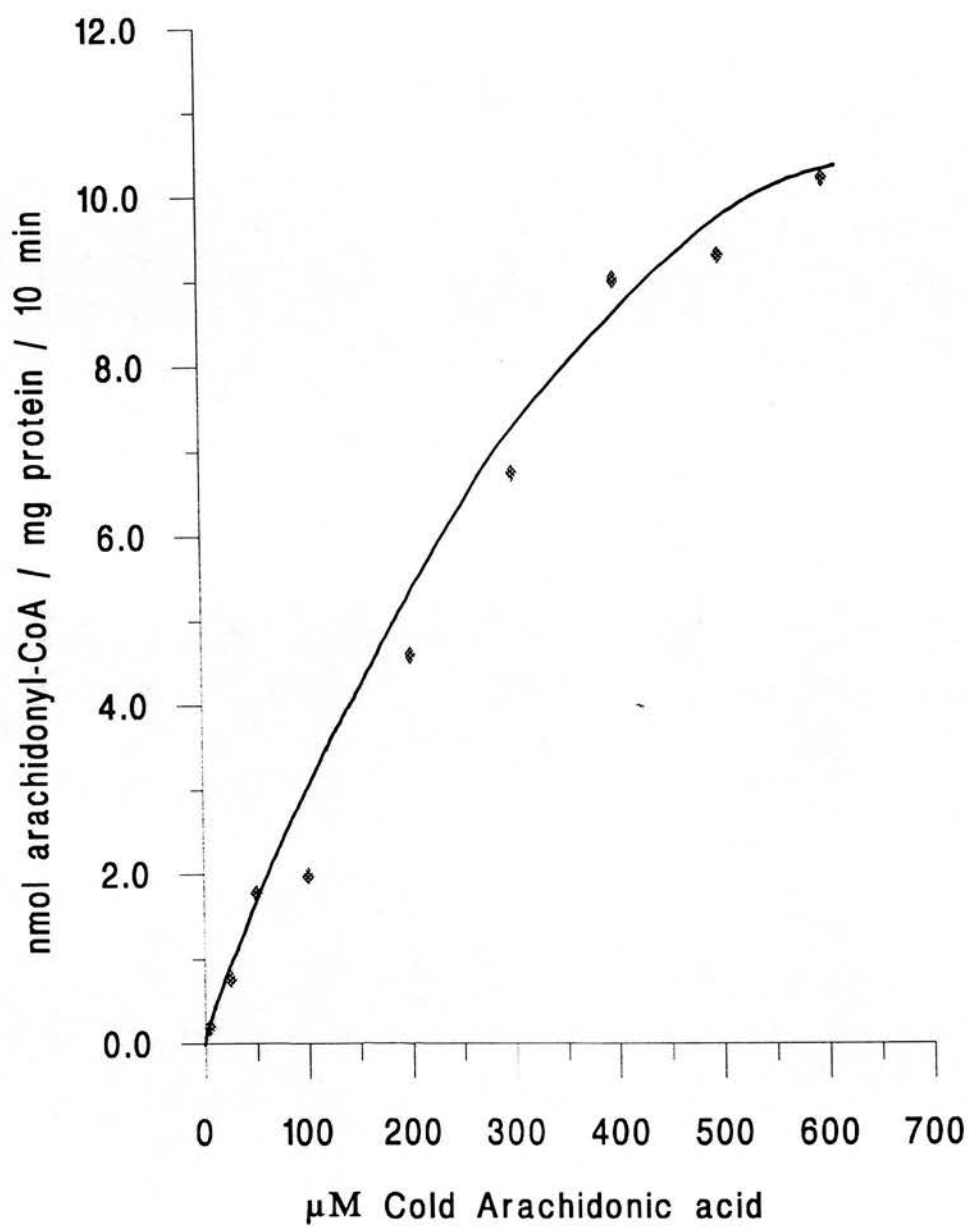
A set of control assay experiments was carried out using non-labelled phospholipid concentrations of 10, 20, 30, 40, 50, 60, 70, 80 and 90  $\mu\text{M}$ . The results are shown in Figure 16. A substrate concentration of 70  $\mu\text{M}$  was used in all further experiments.

### **2:14:3. Incubation time**

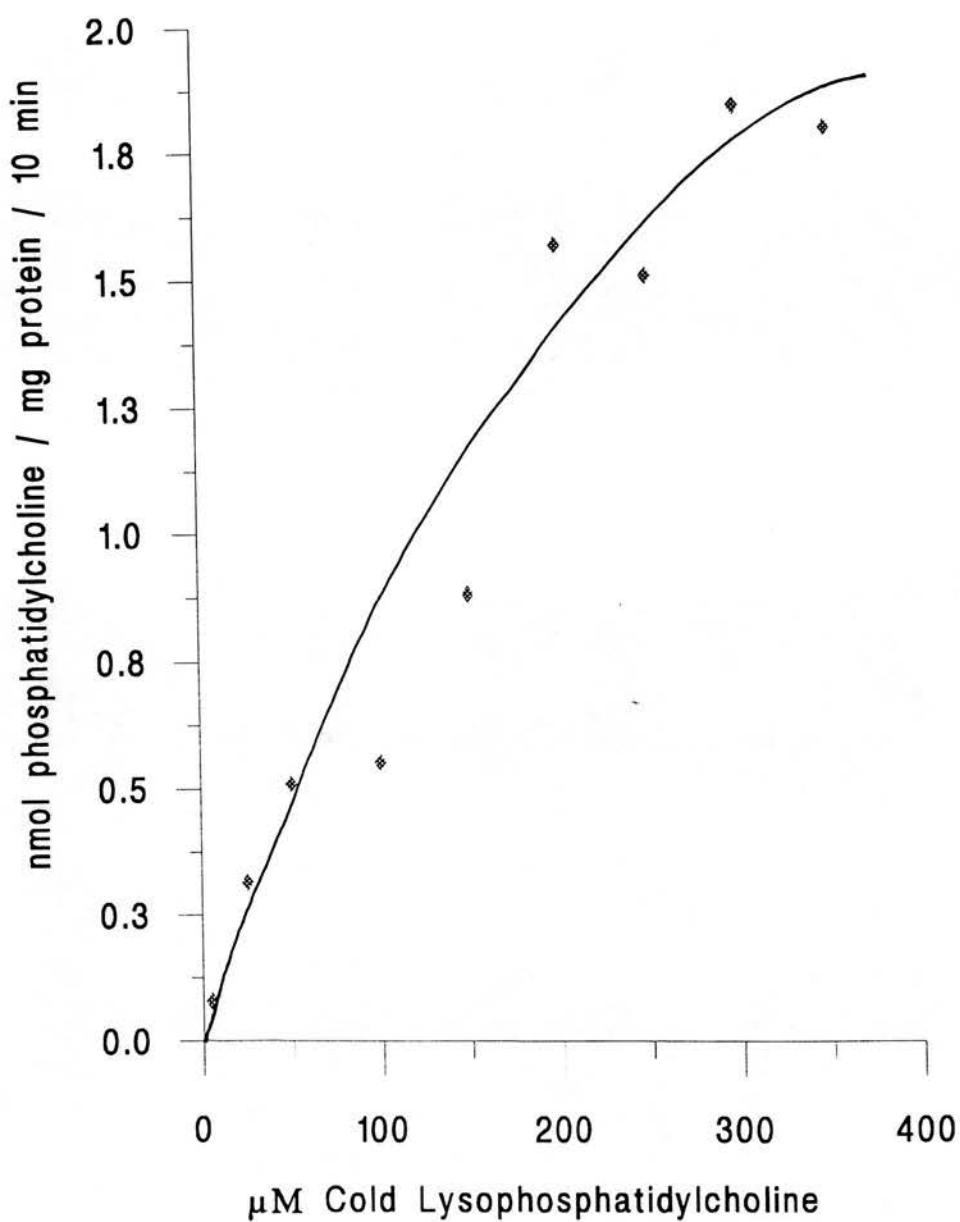
A set of control experiments were carried out for each assay using different incubation times of 1, 2, 5, 8, 10 and 20 min using the appropriate substrate concentrations determined in 2:14:2. The results are shown in Figure 17. It was decided to use an incubation time of 10 min for each assay as this appeared to result in measurable enzyme activity.

### **2:14:4. Protein concentration**

The effect of protein concentration was investigated by diluting the resuspended microsomal pellet in both a series of one-in-two and one-in ten fold dilutions. The results obtained (Figure 18) indicated that the amount of protein in the

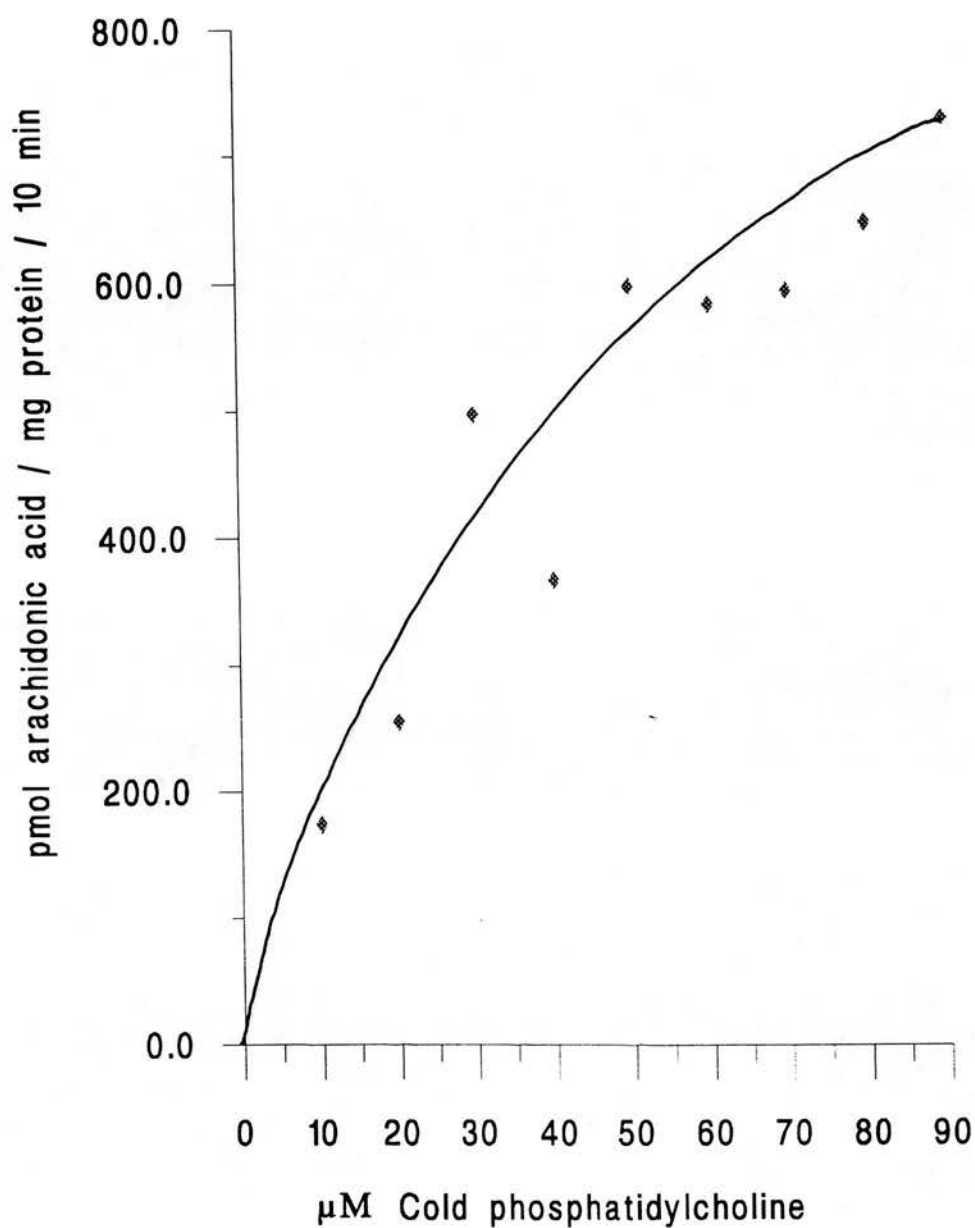


**Figure 14. Effect of non-labelled arachidonic acid concentration on detected acyl-CoA synthetase activity.**

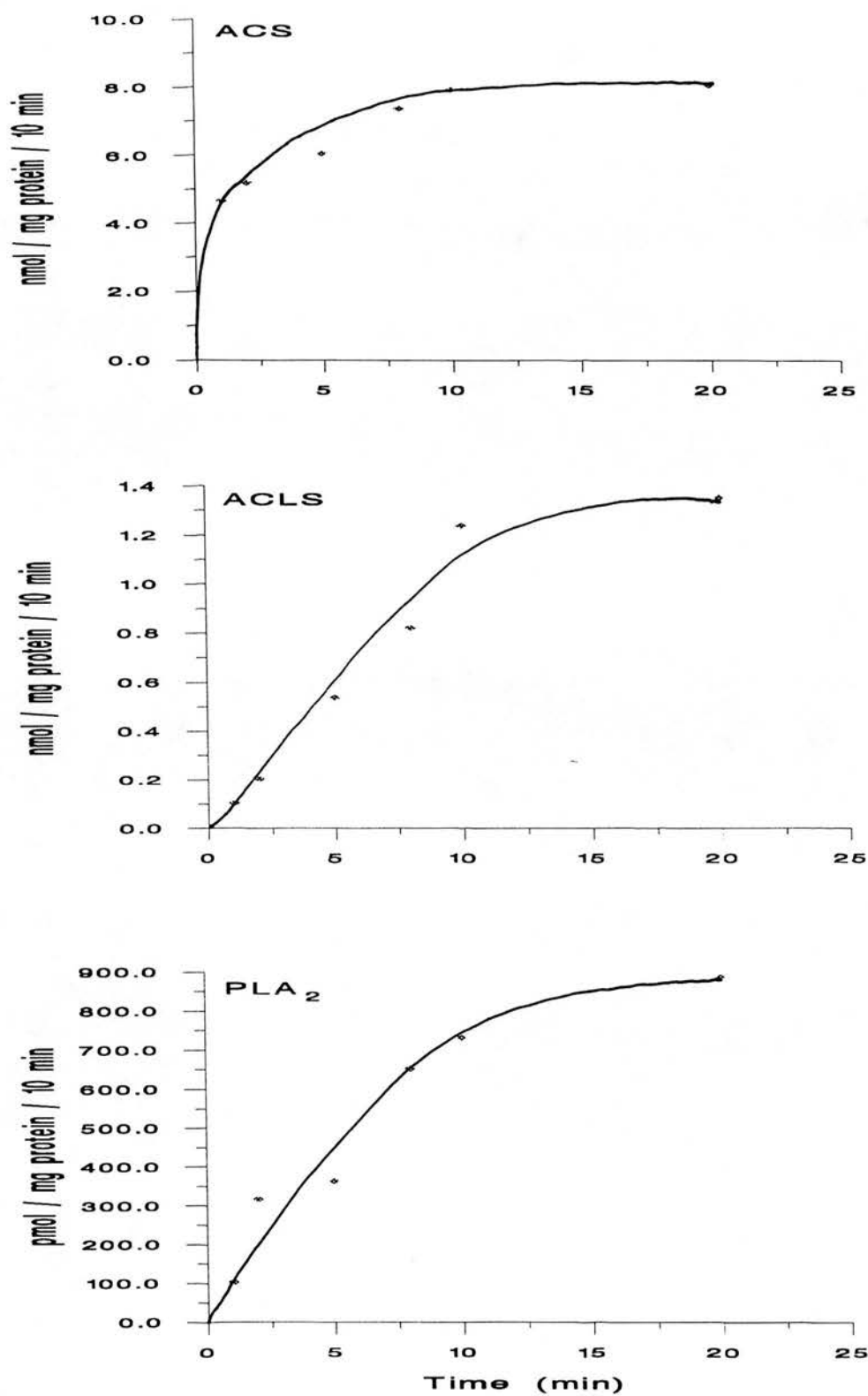


**Figure 15. Effect of non-labelled lysophospholipid concentration on detected acyl-CoA:lysophospholipid acyltransferase activity.**





**Figure 16. Effect of non-labelled phospholipid concentrations on detected phospholipase A<sub>2</sub> activity.**



**Figure 17. Detection of ACS, ACLS and PLA<sub>2</sub> activities with various incubation times.**

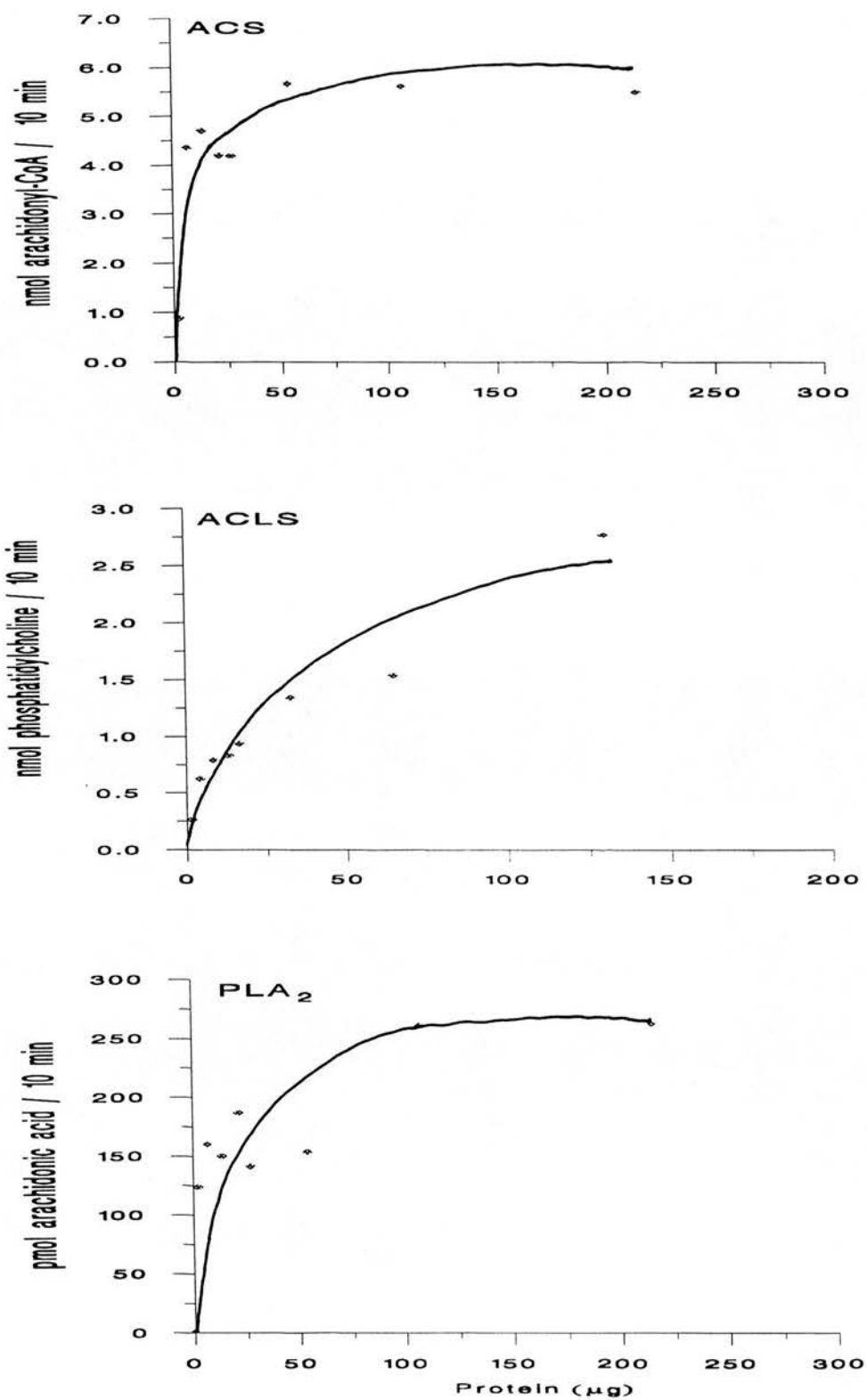


Figure 18. Influence of protein concentration on detected ACS, ACLS and PLA<sub>2</sub> activities.

undiluted microsomal pellet obtained from animals (200-300 µg in 50 µl) resulted in measurable enzyme activity. Therefore protein samples used in the assays were not diluted.

#### **2:14:5. Calcium concentration**

Dependence of the enzyme assays on calcium concentration was investigated in a series of experiments, the results of which are illustrated in Figure 19. The presence of both re-acylating enzymes did not appear to be dependent on calcium; however, PLA<sub>2</sub> presence was enhanced at 7 mM Ca<sup>2+</sup>. Therefore, PLA<sub>2</sub> assays were carried out in the presence of 7 mM Ca<sup>2+</sup>.

### **2:15 Lowry protein assay**

#### **2:15:1 Introduction**

In order to determine the amount of protein present in each microsomal pellet used in each enzyme assay performed, a protein assay was carried out every time. The procedure followed was that of Lowry *et al.* (1951), and is based on the ability of protein molecules to combine with tartrate ions and Folin's phenolic reagent to form a complex which has a spectrophotometric absorbance at 750 nm. The optical absorbance of this complex is proportional to the total amount of protein present in the sample.

#### **2:15:2 Standards and solutions**

Six standard protein solutions were prepared using Bovine serum albumin (BSA). A stock solution of BSA (2.5 mg/ml in distilled water) was prepared from which 0, 0.1, 0.2, 0.3, 0.4 and 0.5 ml were taken and each made up to a total volume of 5 ml with distilled water. Thus, in 0.3 ml of each solution there was 0, 15, 30, 45, 60 and 75 µg protein present, respectively. Stock solutions were prepared as listed below:

Solution A     1 % w/v CuSO<sub>4</sub>.5H<sub>2</sub>O

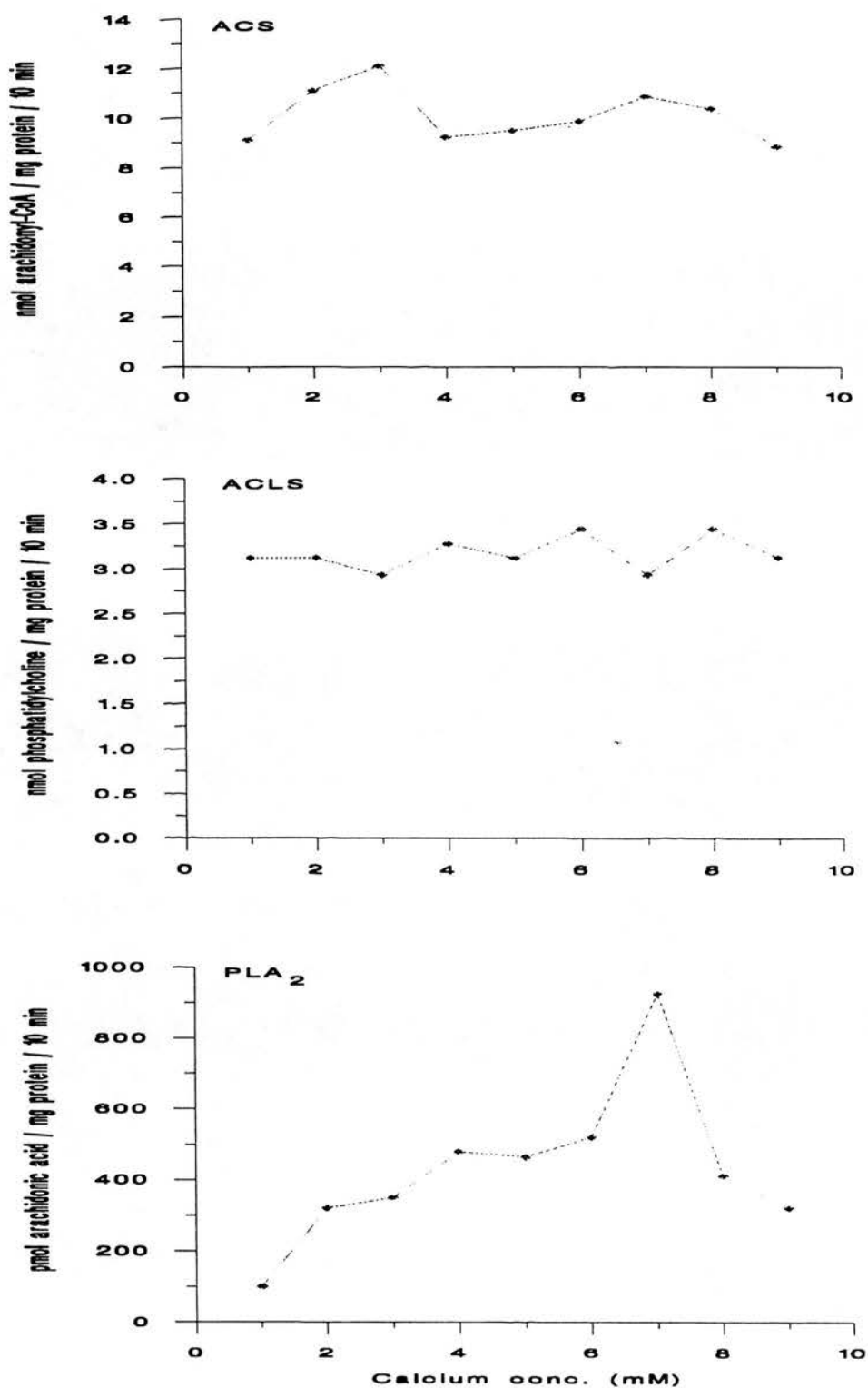
Solution B     2 % w/v NaK tartrate

Solution C     2 % w/v Na<sub>2</sub>CO<sub>3</sub> dissolved in 0.1 M NaOH

Solution D     Folin's phenolic reagent diluted 1:1.5 with distilled water

#### **2:15:3 Assay procedure**

An aliquot of 0.3 ml was removed from each of the six standard protein solutions and placed into six labelled 10 ml test tubes at room temperature. In a beaker, 0.5 ml of Solution A was added to 0.5 ml of Solution B, after which 50 ml Solution C were added and all the solutions mixed. To each of the 0.3 ml standard protein solutions, 3 ml of this A+B+C solution were added and all test tubes



**Figure 19. Influence of calcium concentration on detected ACS, ACLS and PLA<sub>2</sub> activities.**

"whirlmixed". Following a 15 min incubation time (at room temperature), 0.3 ml Solution D was added to each test tube, the tubes "whirlmixed" again, and then allowed to incubate for a further 30 min. The optical absorbance of each protein standard was measured in a Cecil<sup>TM</sup> spectrophotometer at 750 nm wavelength. From the data obtained, a plot of optical absorbance against protein present was constructed.

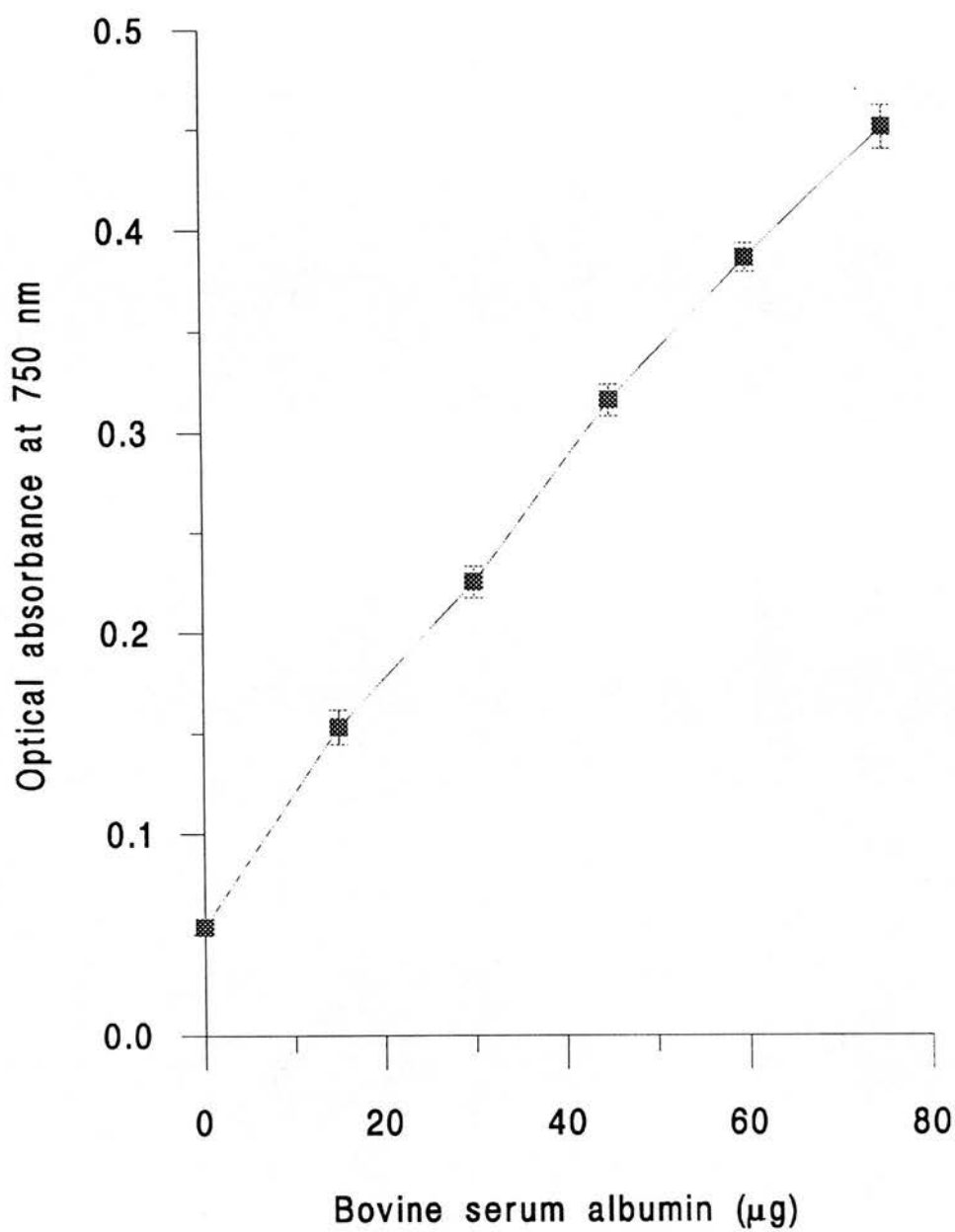
Samples that were to be measured for protein content (having been diluted to a volume of 0.3 ml) were subjected to exactly the same procedure as for the standard solutions. Absorbance values obtained from the unknown samples were applied to the standard plot and the protein content determined. Standard plots were constructed for every protein assay performed to account for any variations between assays. A regression line was constructed (Figure 20) by combining data from several assays carried out. The line had a regression coefficient of 0.99. It was therefore concluded that the method of Lowry *et al.* was a reliable method for measuring protein concentration. To establish that the Lowry assay was linear for the protein (ie. microsomal) solutions used during the assays, a graph was constructed of absorbance against protein concentration of endometrial microsomal protein which had been obtained by a series of one-in-two dilutions. This graph is illustrated in Figure 20a, with the Lowry standard curve for comparison, and illustrates that the Lowry assay did appear to be linear for the microsomal protein solutions. Thus, the method of Lowry was used for measuring protein concentrations in guinea-pig microsomal preparations.

## **2:16 Limitations of methods**

Several points should be considered when interpreting the results observed in the following investigations.

The ability of the Lowry assay to measure protein accurately in the presence of phospholipid has been previously documented (Peterson, 1979). The problem arises from the insolubility of the phospholipids present in the microsomal preparations, which can result in turbid solutions (and thus influence absorbance readings) and interfere with the ability of the protein to react in the aqueous solutions of the Lowry assay. Therefore, protein estimation by the method used in these investigations should be treated with caution, especially since the quantity of phospholipid present in different uterine tissues and at different times of the oestrous cycle and pregnancy, may vary. The problem can be overcome with the addition of sodium dodecyl sulphate (SDS) (Lees and Paxman, 1977; Markwell, Haas, Bieber and Tolbert, 1978; Peterson, 1977, 1979), a powerful negatively charged detergent which





**Figure 20. Standard curve for Lowry protein assay. Each point is the mean  $\pm$  s.e.m. ( n = 5).**

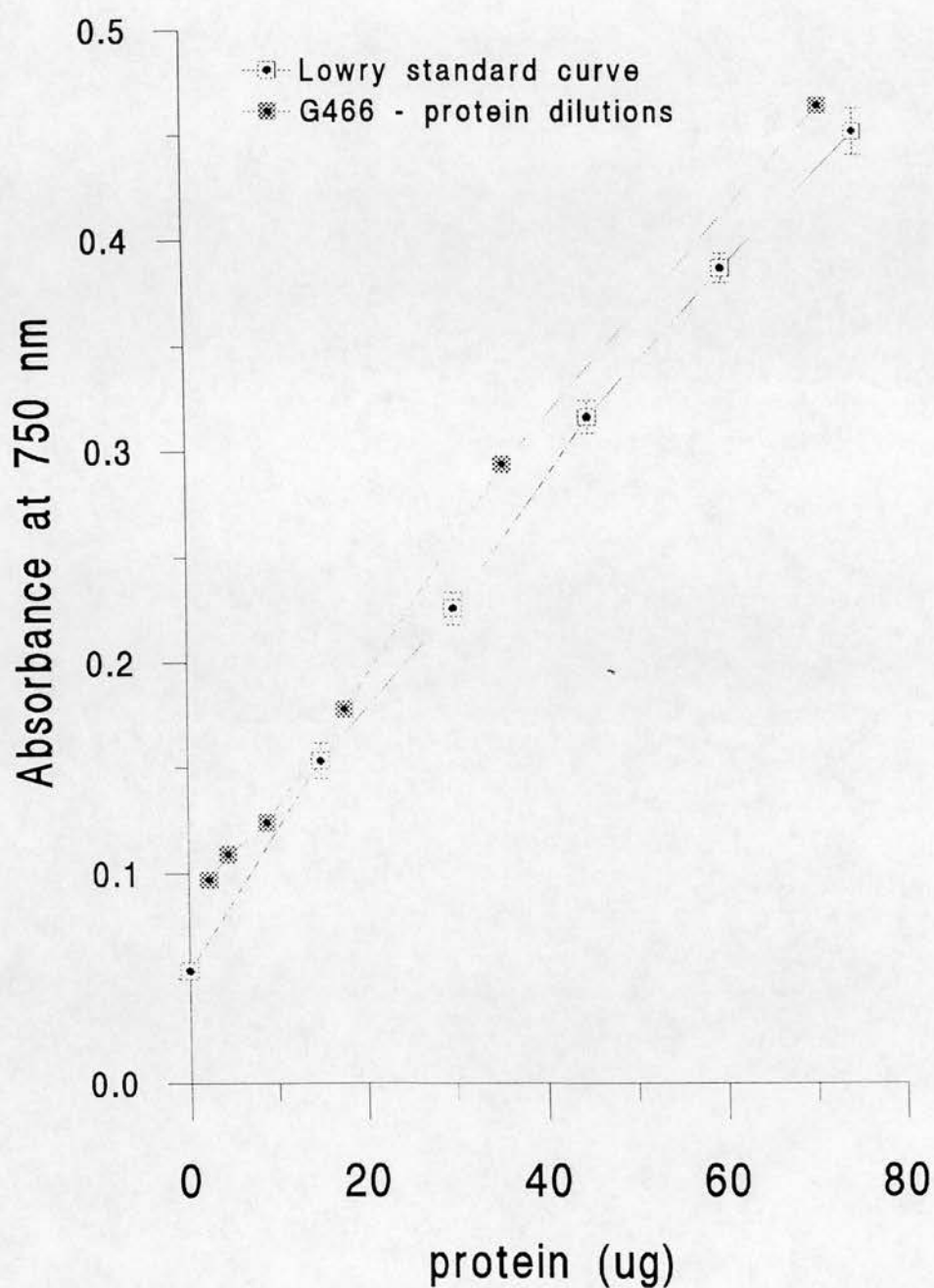


Figure 20a. Lowry assay of a series of one-in-two microsomal protein dilutions ( $n=1$ ) (G466 - protein dilutions), in comparison to standard curve for Lowry protein assay (mean  $\pm$  s.e.m.,  $n=5$ ). The graph would suggest that the Lowry assay was linear for the microsomal protein solutions measured during these investigations.

is added to the reaction mixture dissolving lipids and maintaining them in solution. This detergent binds to hydrophobic regions of molecules causing them to be freed from their associations from other proteins or lipid molecules, and thus render the protein freely soluble in the solution. The Lowry method is also known to give a non-linear calibration curve (Lowry et al., 1951). Therefore, for better precision it is advisable to assay at least three different dilutions of the protein sample. This non-linear behaviour appears to be inherent in the reaction mechanism (Peterson, 1979). Various attempts have been made to improve linearity, either by variation of the reaction conditions or by the application of curve-fitting techniques (Peterson, 1979). It would have been advisable to carry out control Lowry assays with varying dilutions of, not only endometrial microsomal preparations, but of all the different tissue protein solutions (ie. varying dilutions of microsomal preparations from subplacenta, chorio-allantoic placenta and fetal membranes) to investigate whether the Lowry assay was linear for all the different tissue types. Protein/phospholipid content may vary between different uterine and fetal tissues at different times of the oestrous cycle and during pregnancy. The colour given by a protein in the Lowry assay depends on its content of tyrosine and tryptophan residues. Thus, variation of response with various proteins can be quite significant.

In the ACS and PLA<sub>2</sub> assays, the substrate solutions contained phospholipid in aqueous solution. This poses a problem in achieving a homogeneous and properly dissolved substrate solution so that the substrates are accessible to the enzymes. The ACS assay mix contained the detergent Triton X-100 to aid solubilization of the phospholipids. The PLA<sub>2</sub> assay mix was sonicated to disrupt micelle formation. Amphipathic compounds, such as phosphoglycerides, have a hydrophilic head and hydrophobic tail. At an appropriate concentration, the lipid molecules will come together spontaneously to form spheres, termed micelles. The hydrophobic tails interact to exclude water and the charged polar head groups will interact with water and be on the outside of the sphere. The specific concentration of lipid required for micelle formation is referred to as the "critical micelle concentration". Micelles with a single lipid or a mixture of lipids can form. The formation of the micelle depends on the temperature of the system and, if a mixture of lipids are used, on the ratio of concentrations of the different lipids in the mixture. The micelle structure is very stable due to the hydrophobic interaction of the hydrocarbon chains and the attraction of the polar head groups to the water. The critical micelle concentrations for the lipid substrates used in the enzyme assays were unknown. It is not possible to state whether the substrate solutions in the following investigations were homogeneous and fully dissolved. Kinetic studies with PLA<sub>2</sub> are particularly problematic since classical

Michaelis-Menten kinetics do not apply. It is frequently not recognized that enzyme activity increases exponentially when the substrate concentration reaches or exceeds the critical micelle concentration. In addition, PLA<sub>2</sub> can exist in either the inactivated or activated state. The problem of determining enzyme activity is further compounded by the fact that the enzyme may exist in solution as monomers or dimers of varying stability. Unless experimental conditions are well-defined, these differences illustrate some of the problems encountered with the study of this enzyme (Chang, Musser and McGregor, 1987).

Microsomes are membranous vesicles formed by the fragmentation of the endoplasmic reticulum. The precise location of ACS, ACLS and PLA<sub>2</sub> on the endoplasmic reticulum (ie. inside, outside) in guinea-pig uterine and fetal tissues is unknown. These enzymes may have differing positions, and thus have differing abilities to react with exogenous substrate during the enzyme assays performed. It is considered that activation of a microsomal (presumably membrane-bound) PLA<sub>2</sub> by intracellular calcium is responsible for controlling the supply of free arachidonic acid for PG synthesis in the guinea-pig uterus during the oestrous cycle (Downing and Poyser, 1983). In calf thymus lymphocytes and rabbit lymphocytes, ACLS activity is located preferentially in the plasma membrane. However, in other cells, such as liver, much of the activity resides in the endoplasmic reticulum (Szamel and Resch, 1973, 1981). In rat heart myocardial cells, ACLS activity is also localized in microsomes (Severson and Fletcher, 1984). ACS and ACLS activities are usually ascribed to the microsomal or mitochondrial fraction of cellular homogenates, however rabbit cardiac cytosol has also been demonstrated to contain both ACS and ACLS necessary for phospholipid synthesis (Needleman, Wyche, Sprecher, Elliott and Evers, 1985).

In the ACS assay, control cold substrate experiments were only carried out to investigate the effects of different concentrations of arachidonic acid on the enzyme activity. However, CoA can also be considered as a substrate for ACS and thus CoA concentration will also influence the activity of ACS. Therefore, for measurement of ACS activity it is also necessary to determine the optimum concentration of CoA required for the enzyme assay. When establishing an enzyme assay, the Michaelis-Menten constant ( $K_m$ ) should be measured for all substrates. The  $K_m$  is equivalent to the substrate concentration at which  $V_o$  (the initial velocity) is equal to one-half of the  $V_{max}$  (maximum velocity) at steady-state kinetics. The  $K_m$  is measured by a plot of  $V_o$  (enzyme activity) versus the substrate concentration ( $[S]$ ) (approaching a rectangular hyperbola), or through a linear Lineweaver-Burk plot ( $1/[S]$  versus  $1/V_o$ ). The line will have a slope of  $K_m/V_{max}$ , an intercept of  $1/V_{max}$  on the  $1/V_o$  axis, and an intercept of  $-1/K_m$  on the  $1/[S]$  axis. Unfortunately the  $K_m$  was not determined during these

investigations (or  $V_{\max}$ , since insufficient  $[S]$ s were tested,  $V_{\max}$  was not clearly reached). Optimum substrate concentrations should ideally represent at least 10 times the substrate concentration that results in the  $K_m$  value for a particular assay. This was not the case in these investigations. Using such substrate concentrations will ensure that the enzyme is not limited by a lack of substrate.

An appropriate concentration of enzyme (protein) must also be established for use in an enzyme assay in order for accurate quantitative determination of enzyme activity. A concentration should be selected which results in steady-state enzyme kinetics, where the enzyme activity is linearly proportional to the quantity of enzyme used. In control experiments carried out in section 2:14:2, an inappropriate concentration of enzyme was selected for further enzyme assays, since this concentration did not lie on the linear part of the curve. By using enzyme concentrations that lie on the linear part of the standard curve, different enzyme assays can be assumed to have similar steady-state kinetics and thus their activities can be directly related. With varying quantities of enzyme present in different assays, enzyme activity can be detected, but comparisons cannot be made between activities, since reaction rates will vary meaning that enzyme activity cannot be directly related to the concentration of enzyme present.

Due to the above reasons, the ACS, ACLS and PLA<sub>2</sub> assays performed are able to establish the presence of, but not changes in, the enzyme activities investigated.

## CHAPTER 3: RESULTS

### INVESTIGATIONS INTO THE PRESENCE OF ACYL-CoA SYNTHETASE, ACYL-CoA:LYSOPHOSPHOLIPID ACYLTRANSFERASE AND PHOSPHOLIPASE A<sub>2</sub> IN THE GUINEA-PIG UTERUS

#### **3:1 Investigation into the presence of arachidonyl-CoA synthetase (ACS), acyl-CoA:lysophospholipid acyltransferase (ACLS) and phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in day 7 and day 15 non-pregnant guinea-pig endometrium.**

##### **Introduction**

There is much evidence that PGF<sub>2α</sub> is the uterine luteolytic hormone in the guinea-pig (Poyser, 1976). The output of PGF<sub>2α</sub> from the guinea-pig uterus increases approximately 20-fold between days 7 and 15 of the oestrous cycle when measured either *in vivo* or *in vitro* (Blatchley *et al.*, 1972; Poyser and Brydon, 1983). The release of free arachidonic acid from some bound source is regarded as the rate limiting step in PG synthesis by tissues. In the guinea-pig uterus 93 % of the total arachidonic acid present is bound to phospholipids, of which 80 % is bound to PC and PE (Leaver and Poyser, 1981). This bound arachidonic acid is readily releasable by PLA<sub>2</sub> (Downing and Poyser, 1983). In the guinea-pig uterus a refractoriness is observed following repeated stimulation with PLA<sub>2</sub>, but not with arachidonic acid which always induces maximum PG synthesis (Poyser, 1991). This refractoriness in uterine PGF<sub>2α</sub> synthesis may be due to the depletion of the arachidonic acid pool esterified to phospholipids. This arachidonic acid pool would appear to be readily releasable for PG synthesis, but requires 3-5 h to be refilled (Poyser, 1991). The rate of incorporation of arachidonic acid into phospholipids of the guinea-pig endometrium has been shown to be 2- to 3-fold higher on day 15 than on day 7 of the oestrous cycle (Ning *et al.*, 1983). Therefore, the mechanisms controlling arachidonic acid incorporation into phospholipids may be as essential as those which control arachidonic acid release from phospholipid stores in the regulation of endometrial



PGF<sub>2α</sub> synthesis. The three enzymes, ACS, ACLS and PLA<sub>2</sub> control arachidonic acid turnover via deacylation (PLA<sub>2</sub>) and reacylation (ACS and ACLS) of glycerophospholipids. Consequently, all three enzymes may be important in controlling the supply of free arachidonic acid for conversion into PGF<sub>2α</sub> within the endometrium. Therefore, the presence of all three enzymes has been investigated in day 7 and day 15 (days of low and high PGF<sub>2α</sub> output from the uterus, respectively) non-pregnant guinea-pig endometrium.

## Methods

The uteri from five day 7, and five day 15 non-pregnant guinea-pigs were removed. The two uterine horns were immediately separated and placed into ice-cold Tris-HCl buffer (one horn into 0.05 M, pH 8.0 for ACS and PLA<sub>2</sub> assays, and the other horn in 0.05 M, pH 7.5 for ACLS assay). Each uterine horn was opened longitudinally and the endometrium dissected away from the myometrium by cutting away 1-2 mm<sup>3</sup> sections of endometrium using a pair of fine scissors. The endometrial tissue from each horn was then homogenised separately in the appropriate Tris buffer using a ground glass homogeniser, followed by sonication to give a 25 % homogenate. From these homogenates, two microsomal pellets were prepared as described in section 2:10.

### ACS assay

The microsomal pellet prepared was resuspended in 0.25 ml Tris-HCl, pH 8.0. Two 10 µl aliquots were removed and used for the estimation of protein content by the method of Lowry *et al.* (1951) as described in section 2:15. The enzyme assay was performed in duplicate as described in section 2:11. The cold fatty acid used was arachidonic acid along with [<sup>3</sup>H]-arachidonic acid in order to measure specific arachidonyl-CoA synthetase activity. The activity of acyl-CoA-synthetase was calculated as the conversion of [<sup>3</sup>H]-fatty acid to [<sup>3</sup>H]-fatty acid-CoA during a 10 min incubation period as a percentage of the total radioactivity recovered.

### ACLS Assay

The microsomal pellet prepared was resuspended in 0.25 ml Tris-HCl, pH 7.5. Two 10  $\mu$ l aliquots were removed and used for the estimation of protein content by the method of Lowry *et al.* (1951).

ACLS activity was measured using a modified version of the procedures described by Severson and Fletcher (1984) and Fuse, Iwanaga and Tai (1989). The conversion of labelled lysophosphatidylcholine to phosphatidylcholine in the presence of donor arachidonyl-CoA was measured as described in section 2:12. The ACLS activity was routinely determined in duplicate in eppendorf tubes in 10 min assay incubations containing 250  $\mu$ M cold L- $\alpha$ -lysophosphatidylcholine-palmitoyl (LPC) with 0.1  $\mu$ Ci (1.78 nmol) L-lyso-3-phosphatidylcholine-1[1- $^{14}$ C]-palmitoyl, 20 nmol arachidonyl-CoA, 5 mM MgCl<sub>2</sub> and a source of enzyme (50  $\mu$ l resuspended microsomal pellet). The reaction was initiated by the addition of the enzyme and incubated at 37°C for 10 min, after which the reaction mixture was immediately placed into a 10 ml glass centrifuge tube containing 3 ml CHCl<sub>3</sub>:CH<sub>3</sub>OH (2:1 v/v) with 0.05% antioxidant to terminate the reaction. 0.1 M CaCl<sub>2</sub> (1 ml) was also added and the mixture vortexed vigorously. The aqueous and solvent phases were separated by centrifugation at 1000 x g for 5 min. The lower phase was dried under a stream of N<sub>2</sub>, resuspended in 50  $\mu$ l of CHCl<sub>3</sub>:CH<sub>3</sub>OH (2:1 v/v) and stored at -20°C. Lysophosphatidylcholine and phosphatidylcholine fractions (labelled and unlabelled) were separated by thin layer chromatography on glass thin layer silica gel chromatographic plates. ACLS activity was calculated as the conversion of [ $^{14}$ C]-LPC to [ $^{14}$ C]-PC during a 10 min incubation, as a percentage of the total radioactivity recovered.

### PLA<sub>2</sub> Assay

The microsomal pellet resuspended in 0.25 ml Tris-HCl, pH 8.0 for the ACS assay was also used as a source of enzyme for the PLA<sub>2</sub> assay. The procedure used for determination of PLA<sub>2</sub> activity was based on the method described originally by Consentino and Ellis (1981) and modified by Downing and Poyser (1983). The assay

was carried out in duplicate using 50 µl aliquots of resuspended microsomal pellet and phosphatidylcholine with [ $^{14}\text{C}$ ]-arachidonic acid in the carbon-2 position (0.1 µCi) as the labelled substrate as described in section 2:13. The unmetabolised substrate, [ $^{14}\text{C}$ ]-PC, and the fatty acid, [ $^{14}\text{C}$ ]-AA, were separated on silica gel columns using two different solvent systems. [ $^{14}\text{C}$ ]-AA was eluted in hexane/1,4-dioxan/glacial acetic acid (85:15:1) and [ $^{14}\text{C}$ ]-PC was eluted using the more polar solvent, chloroform/methanol/water (65:35:4). The radioactivity in each solvent fraction was measured by counting in a liquid scintillation counter for 4 min. The PLA<sub>2</sub> activity was calculated as the conversion of [ $^{14}\text{C}$ ]-PC to [ $^{14}\text{C}$ ]-AA during a 10 min incubation period calculated as a percentage of the total radioactivity recovered.

ACS, ACLS and PLA<sub>2</sub> assays were carried out at both high and low substrate concentrations. These concentrations are listed in the table below:-

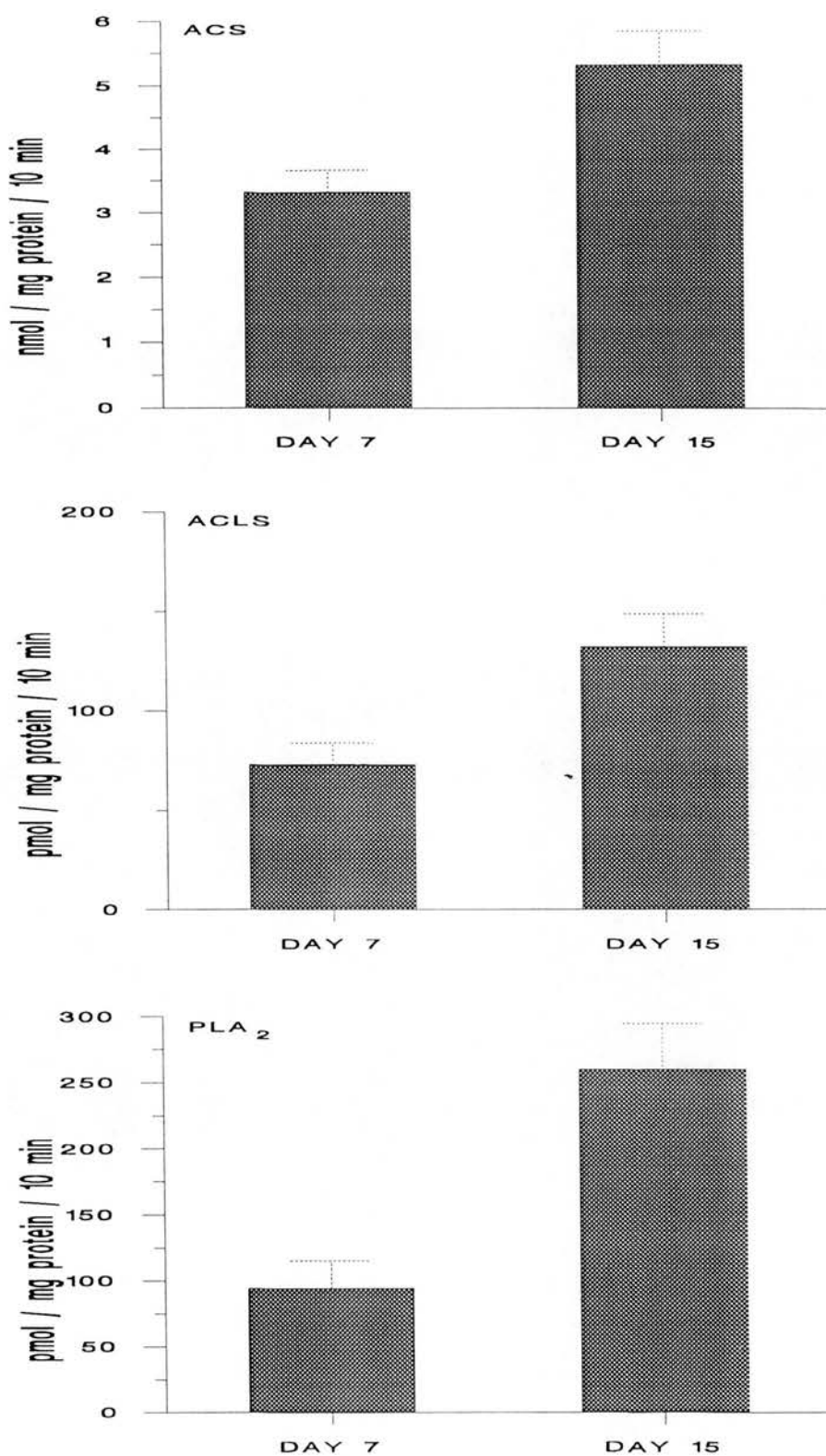
ASSAY	LOW SUBSTRATE CONCENTRATION	HIGH SUBSTRATE CONCENTRATION
ACS	100 µM	500 µM
ACLS	25 µM	250 µM
PLA <sub>2</sub>	16 µM	70 µM

## Results

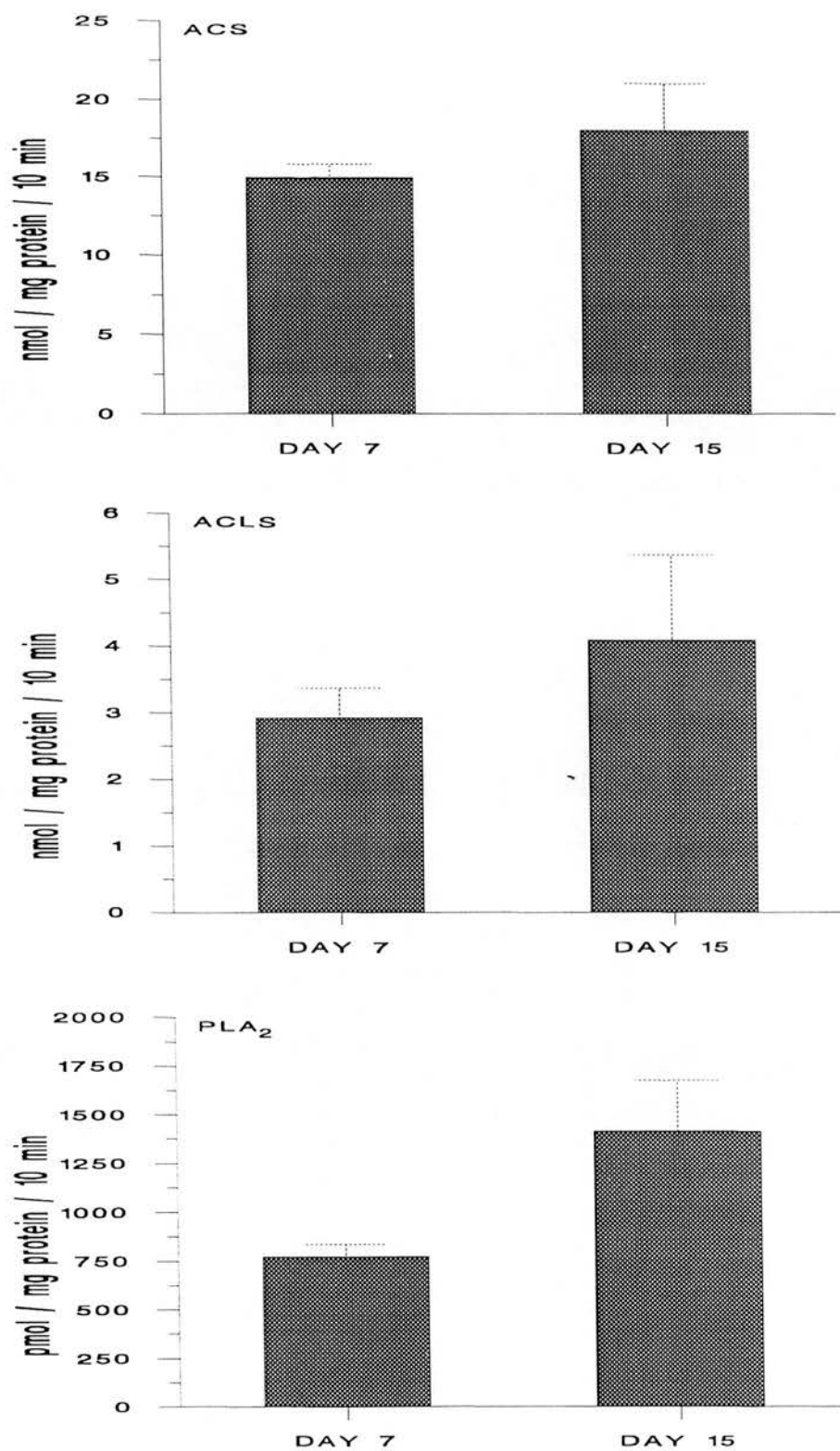
For the reasons listed in section 2:16, the assays carried out were only able to establish the presence of, but not changes in, the activities of ACS, ACLS and PLA<sub>2</sub>.

Detected ACS, ACLS and PLA<sub>2</sub> activities, in day 7 and day 15 non-pregnant guinea-pig endometrium at low and high substrate concentrations, are illustrated in Figures 21 and 22, respectively.

ACS, ACLS and PLA<sub>2</sub> activities were all detected in the endometrium of day 7 and day 15 non-pregnant guinea-pigs, at both low and high substrate concentrations.



**Figure 21.** Mean ( $\pm$  s.e.m.,  $n=5$ ) detected activities of ACS, ACLS and PLA<sub>2</sub> at low substrate concentration in day 7 and day 15 guinea-pig endometrium.



**Figure 22.** Mean ( $\pm$  s.e.m.,  $n=5$ ) detected activities of ACS, ACLS and PLA<sub>2</sub> at high substrate concentration in day 7 and day 15 guinea-pig endometrium.

## Discussion

The three enzymes, ACS, ACLS and PLA<sub>2</sub>, which control arachidonic acid turnover via deacylation (PLA<sub>2</sub>) and reacylation (ACS and ACLS) of glycerophospholipids, were detected to be present in the endometrium of both day 7 and day 15 non-pregnant guinea-pigs. Unfortunately, due to the limitations of the assay methods (see section 2:16), changes in the activities of the enzymes in the endometrium at different times of the oestrous cycle, could not be measured. Therefore, whether these enzymes have a role in the control of arachidonic acid turnover towards the end of the oestrous cycle, coincident with the large increase in PGF<sub>2α</sub> production by the endometrium which occurs at this time, is unknown.

The activation of PLA<sub>2</sub> by calcium is considered to be responsible for the release of arachidonic acid from phospholipids in the guinea-pig endometrium towards the end of the cycle (Downing and Poyser, 1983). Stimulation of PLA<sub>2</sub> activity would lead to an increase in the concentration of free arachidonic acid released from phospholipid stores and therefore an increase in endometrial PGF<sub>2α</sub> production. Endometrial ACS and ACLS activities could possibly be specifically stimulated towards the end of the cycle or could increase as a consequence of an increase in the concentration of free arachidonic acid released by PLA<sub>2</sub> which needs to be "mopped up" and taken back into phospholipids.

Oestradiol acting on a progesterone-primed uterus is considered to be the physiological stimulus for increased PGF<sub>2α</sub> production by the guinea-pig uterus (particularly the endometrium) towards the end of the oestrous cycle (Blatchley *et al.*, 1972; Earthy *et al.*, 1975; Antonini *et al.*, 1976). In the following experiment, the presence of ACS, ACLS and PLA<sub>2</sub> activities in the endometrium of ovariectomized guinea-pigs treated with oestradiol and/or progesterone, or vehicle-treated, has been investigated. All further enzyme assays were carried out at a high substrate concentration.



### **3:2     Investigations into the presence of arachidonyl-CoA synthetase, acyl-CoA:lysophospholipid acyltransferase and phospholipase A<sub>2</sub> in the endometrium of ovariectomized guinea-pigs treated with oestradiol and/or progesterone.**

#### **Introduction**

During the guinea-pig oestrous cycle, oestradiol secretion from the ovary increases from day 10 (Joshi *et al.*, 1973) which precedes the increase in PGF<sub>2α</sub> secretion from the uterus by 24 h (Blatchley *et al.*, 1972; Earthy *et al.*, 1975; Antonini *et al.*, 1976). Oestradiol administered to ovariectomized guinea-pigs maintained on progesterone stimulates uterine PGF<sub>2α</sub> output (Blatchley and Poyser, 1974; Poyser, 1983b). Also, the administration of onapristone (a progesterone antagonist) or ICI 182780 (an oestrogen antagonist) to non-pregnant guinea-pigs on days 11-14 of the cycle significantly reduces uterine PGF<sub>2α</sub> output on day 15 (Poyser, 1993). Therefore oestradiol acting on a progesterone-primed uterus appears to be the physiological stimulus for increased secretion of endometrial PGF<sub>2α</sub>, especially as oxytocin has no stimulatory effect on endometrial PGF<sub>2α</sub> synthesis in guinea-pigs (Poyser and Brydon, 1983; Riley and Poyser, 1987). It has been proposed that oestradiol may have a dual action increasing uterine PG synthesizing capacity and directing synthesis toward PGF<sub>2α</sub> (Poyser, 1983b). Whether oestradiol and progesterone can control endometrial PG synthesis by controlling the necessary supply of free arachidonic acid towards the end of the cycle is not known. The following experiment was carried out to investigate whether the enzymes that control arachidonic acid turnover (ACS, ACLS and PLA<sub>2</sub>) were present in the endometrium of ovariectomized guinea-pigs treated with oestradiol and/or progesterone.

#### **Methods**

Twenty guinea-pigs weighing approximately 600 g, which had normal oestrous cycles, were ovariectomized during the luteal phase of the cycle. The guinea-pigs were anaesthetized by injection of 30 mg/kg sodium pentobarbitone, and a dorsal incision was made on either side of the backbone. The Fallopian tubes were ligated in

two places and severed between the ligatures, enabling the ovaries to be removed. To confirm that the guinea-pigs had undergone successful ovariectomy, the animals continued to be examined daily to check that they no longer displayed oestrous cycles. Eight weeks later, the guinea-pigs were divided randomly into four groups of five animals and received subcutaneous injections of arachis oil vehicle (vehicle) containing the following additions:-

- |         |                                                                                                                                                                         |
|---------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Group 1 | No additions - 0.5 ml vehicle daily for 10 days.                                                                                                                        |
| Group 2 | 0.5 ml vehicle daily for 7 days followed by 0.5 ml vehicle containing 10 µg oestradiol benzoate daily for 3 days.                                                       |
| Group 3 | 0.5 ml vehicle containing 2.5 mg progesterone daily for 10 days.                                                                                                        |
| Group 4 | 0.5 ml vehicle containing 2.5 mg progesterone daily for 7 days, followed by 0.5 ml vehicle containing 10 µg oestradiol benzoate + 2.5 mg progesterone daily for 3 days. |

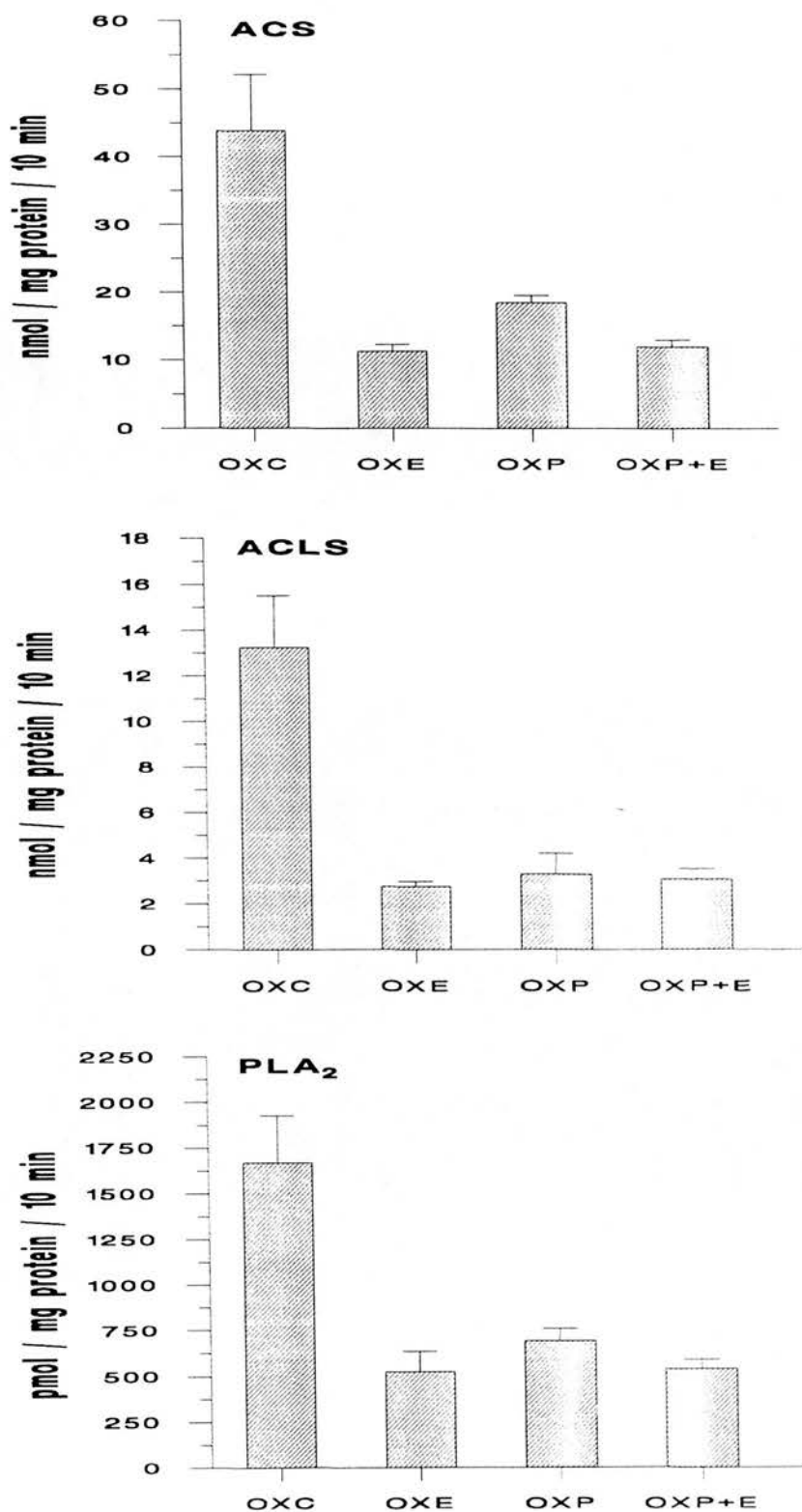
All guinea-pigs were injected between 09:00 and 09:30 h. On the 11th day after starting treatment, each guinea-pig was killed by stunning and incision of the neck. The uterus was removed and separated into its two uterine horns. ACS, ACLS and PLA<sub>2</sub> assays were then carried out using exactly the same methods as described in experiment 3:1.

### Statistical Tests

The differences between the uterine weights of ovariectomized guinea-pigs treated with vehicle, oestradiol, progesterone and oestradiol + progesterone were analysed by Student's t-test.

### Results

For the reasons listed in section 2:16, the assays carried out were only able to establish the presence of, but not changes in, the activities of ACS, ACLS and PLA<sub>2</sub>. Detected ACS, ACLS and PLA<sub>2</sub> in the endometrium of ovariectomized guinea-pigs treated with oestradiol and/or progesterone are shown in Figure 23. All three enzymes



**Figure 23.** Mean ( $\pm$  s.e.m.,  $n=5$ ) detected activities of ACS, ACLS and PLA<sub>2</sub> in the endometrium of ovariectomized (OVX) guinea-pigs treated with vehicle (OXC), oestradiol (OXE), progesterone (OXP), and oestradiol and progesterone (OXP+E).

were detected in both control (vehicle-treated only) and treated, (oestradiol and/or progesterone-treated), ovariectomized guinea-pig endometrium.

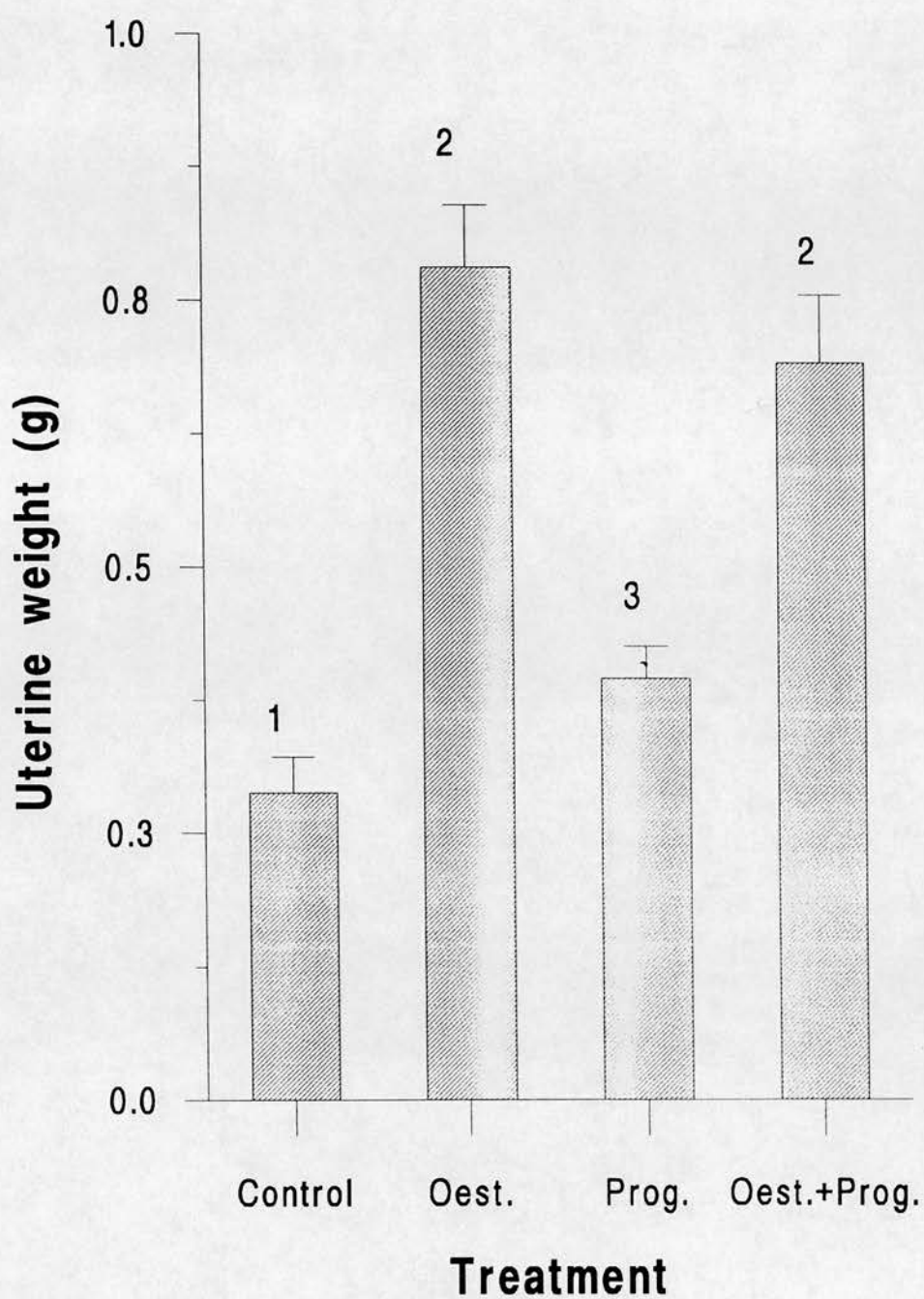
Figure 24 illustrates the influence of progesterone and/or oestradiol treatment on the uterine weight of ovariectomized guinea-pig uterus. It is clear that both oestradiol and progesterone treatment used in this experiment significantly increased uterine weight, with oestradiol having the major effect.

## Discussion

The significant increase in uterine weight in ovariectomized animals following steroid treatment suggests that the combined effects of oestradiol and progesterone on the uterus are to prepare the uterus for implantation and pregnancy.

ACS, ACLS and PLA<sub>2</sub> activities were found to be present in the endometrium of control and steroid-treated ovariectomized guinea-pigs and thus, may have a role in the control of arachidonic acid turnover and PG synthesis in the guinea-pig endometrium. Due to the limitations of the assay methods (see section 2:16), changes in the activities of the enzymes in the endometrium of ovariectomized animals following steroid treatment, could not be measured. Therefore, whether these endometrial enzymes are influenced by the action of oestradiol acting on a progesterone-primed uterus (considered to be the physiological stimulus for increased PGF<sub>2α</sub> synthesis towards the end of the oestrous cycle) is unknown.

If an increase in the rate of arachidonic acid uptake (reacylation) back into phospholipids by ACS and ACLS were to occur, this could be beneficial in providing a faster turnover of arachidonic acid for PG synthesis. However, if such an increase did occur, this may actually limit the amounts of PGs synthesized. Following its release by PLA<sub>2</sub>, the amount of free unesterified arachidonic acid available for PG synthesis would be reduced at a faster rate if the uptake processes were stimulated. During early pregnancy, PGF<sub>2α</sub> output from the uterus must be kept low so that luteal regression does not occur and progesterone secretion is maintained. ACS and ACLS may be of importance during this time in the control of available arachidonic acid for PG synthesis. Consequently, the presence of ACS, ACLS and PLA<sub>2</sub> in early pregnant guinea-pigs has been investigated in the following experiment.



**Figure 24.** Mean ( $\pm$  s.e.m.,  $n=5$ ) uterine weights of ovariectomized guinea-pigs treated with vehicle (Control), oestradiol (Oest.), progesterone (Prog.), oestradiol and progesterone (Oest.+Prog.). Columns with the same number are not significantly ( $P > 0.05$ ) different.

**3:3. Investigations into the presence of acyl-CoA synthetase, acyl-CoA:lysophospholipid acyltransferase and phospholipase A<sub>2</sub> in uterine and conceptual tissues of day 15, 29 and 36 pregnant guinea-pigs.**

**Introduction**

If an animal becomes pregnant, the luteolytic influence of the uterus must be prevented, since continued progesterone secretion is necessary for the whole of gestation in some species (e.g. cow, goat and pig), or during the first third of gestation in others (e.g. sheep and guinea-pig) (Poyser, 1992). In pregnant guinea-pigs, the increase in uterine PGF<sub>2α</sub> output from the uterus after day 11 of the cycle is prevented by the presence of conceptuses and luteal function is therefore maintained (Antonini *et al.*, 1976; Blatchley *et al.*, 1975a, b). It has been proposed that the guinea-pig conceptus secretes an anti-luteolytic factor which may act by suppressing the stimulatory effect of oestradiol on endometrial PGF<sub>2α</sub> synthesis and release (Poyser, 1984a). Conceptuses from several species have been shown to synthesize PGs *in vitro* (Lewis, 1989). The fact that conceptuses produce PGs indicates that conceptuses must influence the control of arachidonic acid turnover and therefore the amount of free arachidonic acid available for PG synthesis. If so, this may provide a mechanism by which the conceptus prevents the release of uterine PGF<sub>2α</sub> during early pregnancy, thus maintaining luteal function and progesterone secretion.

Implantation in the guinea-pig is completed during a period of 6-8 hours on the sixth day following fertilization (Blandau, 1949). The terms “superficial” and “interstitial” describe different implantation mechanisms, and separate those in which the blastocyst remains in the uterine lumen and those in which it penetrates the luminal epithelium and embeds itself in the uterine wall. The guinea-pig blastocyst was first classed as one demonstrating a true type of interstitial implantation by Graf v. Spee in 1883.

The fertilized egg (zygote) of all mammals is surrounded by a mucopolysaccharide coat called the “zona pellucida”. This is transparent, tough and elastic. As the zygote passes along the oviduct (or Fallopian tube, in man), it divides forming a solid clump of cells, known as the morula. The morula is then transformed



into the blastocyst, usually soon after it has reached the uterine lumen. This is a hollow ball of cells with a two-layered wall containing a cavity, referred to as the blastocoele (Perry, 1981). The term “blastocyst” is applicable both before and after the development of the inner layer, or endoderm. The embryo will form from a thicker portion of the ectoderm, known as the embryonic knot or disc. The orientation of the embryonic disc is a species characteristic. The remaining portion of the ectoderm is called the trophoblast, which plays an important role in the formation and structure of the future placenta. The endodermal layer of cells in the blastocyst usually form by tangential division of a few cells of the embryonic disc and then increase around the inner side of the ectoderm surrounding the blastocoele which then becomes the yolk sac. At this stage, the zona pellucida is lost. In some species, the contents are extruded or “hatched” whilst in others the zona may be dissolved by processes that involve lytic enzymes which may originate from either the uterine or embryonic tissues. In the mouse, it has been suggested that PGE<sub>2</sub> produced by the blastocyst promotes water passage across the trophectoderm causing the blastocyst to swell and the zona to burst (Biggers, Leonov, Baskar and Fried, 1978).

Once the zona pellucida has been lost, the fetal membranes are developed. These comprise the amnion, yolk sac, chorion and allantois, all of which are of embryonic origin, interposed between embryo and mother. These structures, together with the fetus, can be referred to collectively as the conceptus. The placenta is formed when fetal tissues contact or fuse with maternal tissue for physiological exchange; in mammals, this always involves the chorion, and either the yolk sac or the allantois. The amnion remains avascular, and is turgid under the hydrostatic pressure of its contained fluid, providing the “private pond” in which the embryo develops. The development of the fetal membranes is somewhat dependent upon the type of implantation which varies between species. In superficial implantation, the blastocyst normally becomes distended, filling and sometimes swelling the uterine lumen prior to trophoblast attachment to the luminal epithelium. During interstitial implantation, as in the guinea-pig (Spee, 1883) the blastocyst remains small, lodging in a groove or fold of the uterine mucosa where it penetrates the epithelium thus reaching the decidua; a thick cushion created by hypertrophy of the uterine wall at the point of implantation, a

major part of which consists of polyploid cells or “decidual cells”. They are formed in response to the blastocyst in what is referred to as the “decidual cell reaction”.

Implantation in the guinea-pig involves the formation of an “egg cylinder” with an “embryonic pole” and an “ectoplacental pole”. The latter pole contains numerous lacunae which fill with maternal blood as the invasive cells break down the walls of the endometrial capillaries. As the embryo develops, uterine swelling increases due to the distension of the exocoele, the allantois grows out from the ventral surface of the embryo, extends across the exocoele and connects with the mesoderm under the ectoplacental trophoblast to form a chorio-allantois. The ectoplacental trophoblast, and consequently the chorio-allantoic placenta, are directly attached to the mesometrial wall of the uterus, adjacent to the entry of the uterine vessels (Perry, 1981). Implantation in man is also interstitial, but does not involve an ectoplacental mass of trophoblast. The conceptus simply enlarges within the uterine wall at the point of implantation and the overlying tissue constitutes the decidua capsularis.

The guinea-pig has a “haemochorial placenta” which is also found in rat, rabbit and man (Perry, 1981). In this placental type, blood is released from maternal vessels to circulate through channels within the trophoblast before returning to the maternal circulation (Perry, 1981). The guinea-pig has an haemochorial placenta of “labyrinthine” form (Mossman, 1937), whilst in man it is of a “villous” nature (Perry, 1981). The distinction is between a condition where the trophoblast covers vascularized lamellae of fetal mesenchyme (connective tissue of mesodermal origin) and one in which it covers finger-like growths. The guinea-pig conceptus also has a structure known as the “subplacenta”, a specialized area of chorion between the haemochorial placenta and the decidua basalis, which has been described in detail in a study by Davies, Dempsey and Amoroso (1961). This structure was stated to only exist in two animals; the guinea-pig and porcupine, both members of the Hystricomorph group of rodents (Mossman, 1937), however it was later found to be present in other members of this group including the chinchilla and agouti (Perrotta, 1959). The subplacenta, which arises around day 16 of pregnancy, consists of folded lamellae of cytotrophoblast, which is in contact with fetal mesenchyme on its fetal

surface, and which gives rise to syncytial trophoblast on its maternal surface. The subplacenta and chorio-allantoic (haemochorial) placenta are separated by a plane of fetal mesenchyme which passes between the cytotrophoblastic lamellae of the subplacenta and carries vessels to them. The subplacenta merges peripherally with the marginal syncytium of the haemochorial placenta through an "intermediate" zone. An area of necrotic decidua lies deep in the subplacenta through which the vessels supplying the haemochorial placenta pass. During early development, the endothelial walls of these vessels are replaced by invasive trophoblastic elements of the subplacenta. The subplacenta appears to degenerate after day 50 of pregnancy and is necrotic at term. A thin layer of normal decidua lies between the necrotic zone and myometrium; the plane of cleavage of the placenta at term occurs between this layer and the myometrium (Davies *et al.*, 1961).

PGs originating from the conceptus appear to have numerous roles, including, intrauterine migration of embryos, blastocyst hatching from the zona pellucida, ion transport across trophoctoderm, fluid accumulation in the blastocoele, increased endometrial capillary permeability and blastocystic glucose metabolism (see Lewis, 1989). Between days 7 and 12 of pregnancy, pig embryos migrate and become spaced in the uterus. Local myometrial contractions appear to be involved in controlling this migration, and blastocystic PGs may stimulate these contractions (Pope, Maurer and Stormshak, 1982). Before blastocyst implantation, its protective covering, the "zona pellucida" must be shed. It has been demonstrated that inhibitors of PG synthesis and PG antagonists reduce the rate of hatching of mouse embryos from the zona pellucida (Biggers, Leonov, Baskar and Fried, 1978). Since PGE<sub>2</sub> is able to promote water movement across epithelia, it was proposed that PGE<sub>2</sub> produced by the blastocyst promotes water passage across the trophoctoderm resulting in the accumulation of blastocoelic fluid, and the eventual "hatching" of the blastocyst from the zona pellucida (Biggers *et al.*, 1978). Later studies supported this hypothesis (Hurst and Farlane, 1981; Chida, Uehara, Hoshiai and Yajima, 1986). In a study by Lewis (1986b) it was demonstrated that indomethacin decreased PG synthesis by, and transport of, <sup>22</sup>Na across day 16 sheep trophoblast and also prevented the formation of trophoblastic vesicles *in vitro*. This study suggested that PGs may be involved in

the control of fluid accumulation during the period of rapid blastocystic elongation (prior to implantation) in sheep. Localized increases in endometrial capillary permeability precede implantation in numerous mammals, and PGs appear to be involved in initiating these increases (see Lewis, 1989). Other studies have also suggested a role for PGs (possibly of blastocystic origin) in the control of energy metabolism in blastocysts (see Lewis, 1989).

During pregnancy, the conceptus must be able to modulate the maternal humoral (antibody-mediated) and cellular (killer-cell mediated) immune responses. Several mechanisms that have been demonstrated appear to utilize PGE<sub>2</sub>. An immunosuppressive agent produced by cultured human decidual cells has been identified as PGE (Parhar, Kennedy and Lala, 1988).

PGs produced by the endometrium and conceptus appear to be involved in the establishment, maintenance and termination of pregnancy. Therefore, the control of arachidonic acid turnover during pregnancy may be an essential factor in the control of PG synthesis by both the endometrium and conceptus. Consequently, the presence of ACS, ACLS and PLA<sub>2</sub> in uterine and conceptual tissues from day 15, 29 and 36 pregnant guinea-pigs has been investigated.

## Methods

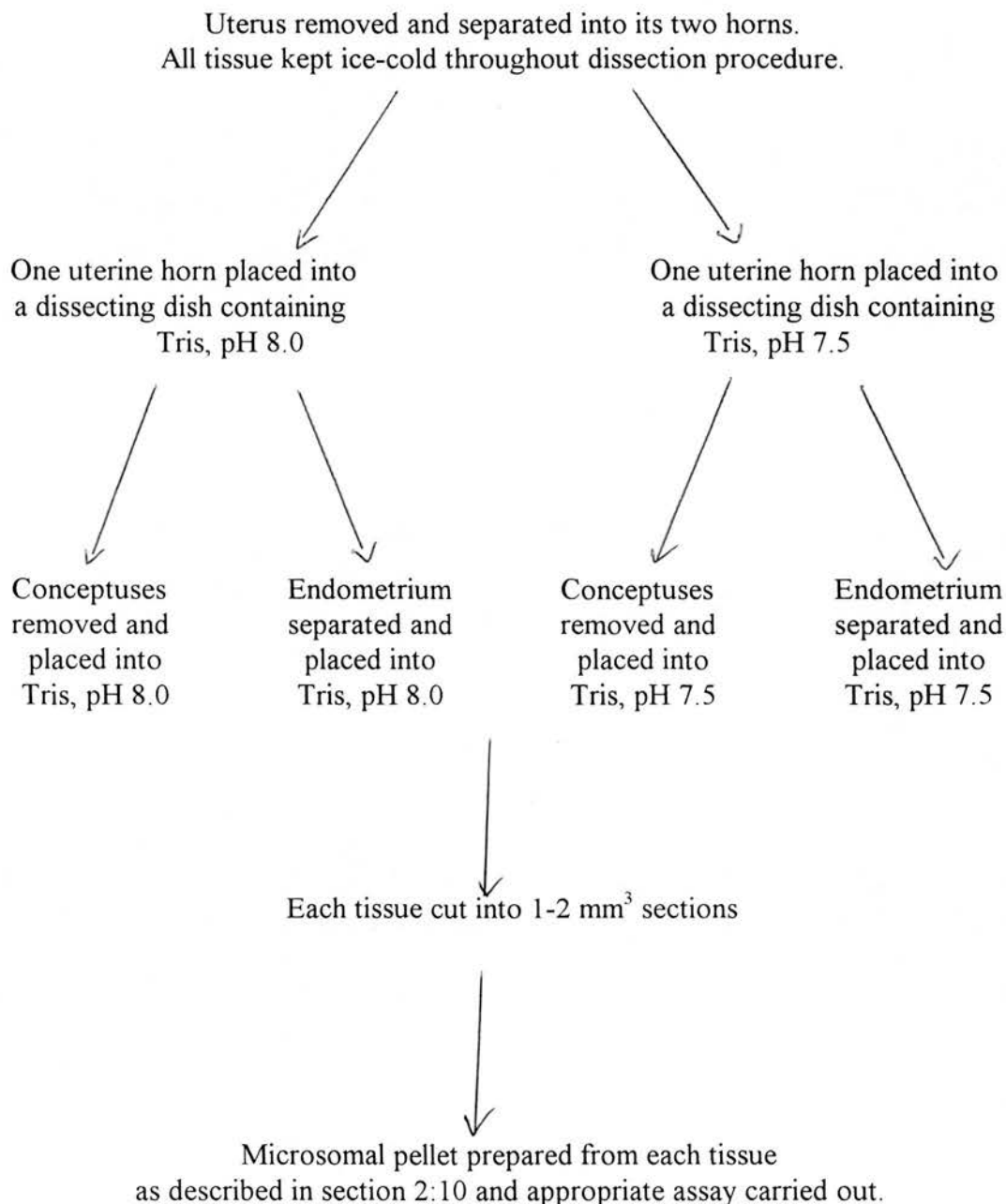
Fifteen virgin female guinea-pigs which had undergone at least 3 normal oestrous cycles were mated by placing them separately on days 14-15 of the cycle (i.e. several days prior to oestrus) with a male guinea-pig of proven fertility. The guinea-pigs were examined daily for signs of mating and for the presence of sperm in the vaginal smear, with day 1 of pregnancy being designated the first day of observing any of these signs.

Day 15 Pregnant: Five guinea-pigs were killed by stunning and incision of the neck on day 15 of pregnancy. The uterus was removed from each animal and separated into its two horns. Each horn was placed into a separate dissecting dish; one containing ice-cold Tris-HCl buffer, pH 8.0 (for ACS and PLA<sub>2</sub> assays) and the other containing ice-cold Tris-HCl buffer, pH 7.5 (for ACLS assay). Each uterine horn was "opened" by a longitudinal incision and the conceptuses removed by gently pulling them away from



the endometrium. The conceptuses were then also placed into separate dishes containing the appropriate Tris-HCl buffer. The endometrium was separated from the myometrium by cutting away 1-2 mm<sup>3</sup> sections of endometrium using a pair of fine scissors. The conceptuses were also cut into 1-2 mm<sup>3</sup> pieces. The general procedure is shown in Figure 25a. The tissue from each dish was homogenised using a ground glass homogeniser followed by sonication to give a 25 % homogenate (250 mg/ml buffer). Four microsomal pellets were prepared from each tissue as described in section 2:10. ACS, ACLS and PLA<sub>2</sub> assays were conducted using exactly the same methods as in experiment 3:1.

Day 29 and 36 Pregnant: Figure 25b shows the general procedure. Five guinea-pigs were killed by stunning and incision of the neck on day 29 and day 36 of pregnancy. The uterus was removed from each animal and separated into its two horns. Each horn was placed into a separate dissecting dish; one containing ice-cold Tris-HCl buffer, pH 8.0 (for ACS and PLA<sub>2</sub> assays) and the other containing ice-cold Tris-HCl buffer, pH 7.5 (for ACLS assay). Each uterine horn was “opened” by a longitudinal incision and the conceptuses, along with the placentae, were removed, by gently pulling them away from the endometrium. Figures 25c, 25d and 25e show photographs taken from a day 36 pregnant guinea-pig, illustrating the uterine horns, conceptuses, and dissection of the placentae. The subplacenta came away very easily from the chorio-allantoic placenta (due to the plane of mesenchyme present between them), which was itself separated from the conceptus by gently snipping the umbilical cord. Separation of the subplacenta was a little more difficult from the decidua, but once separated, the subplacenta appeared as a bulging ovoid structure of firm consistency, greyish in colour, which is in contrast to the deep red colour of the chorio-allantoic placenta. The subplacenta, chorio-allantoic placenta, chorion and amnion were then separated from each conceptus. The two fetal membranes, namely the chorion and amnion, were removed separately by gently pulling each membrane away from the conceptus. The conceptus was then discarded. All tissues were kept ice-cold and cut into 1-2 mm<sup>3</sup> pieces and placed into the appropriate Petri dish prior to homogenisation. The endometrium was separated from the myometrium by cutting away 1-2 mm<sup>3</sup> sections of endometrium using a pair of fine scissors. A microsomal



**Figure 25a.** General procedure for preparing day 15 pregnant guinea-pig conceptual and uterine tissue for the detection of ACS, ACLS and PLA<sub>2</sub> enzyme activity.



Uterus removed and separated into its two horns. All tissue kept ice-cold throughout dissection procedure.



One uterine horn placed into Tris, pH 8.0 and the other uterine horn placed into Tris, pH 7.5



Each uterine horn dissected to separate the endometrium from the myometrium, and to separate the subplacenta, chorio-allantoic placenta, chorion and amnion from the conceptus.



Conceptuses discarded



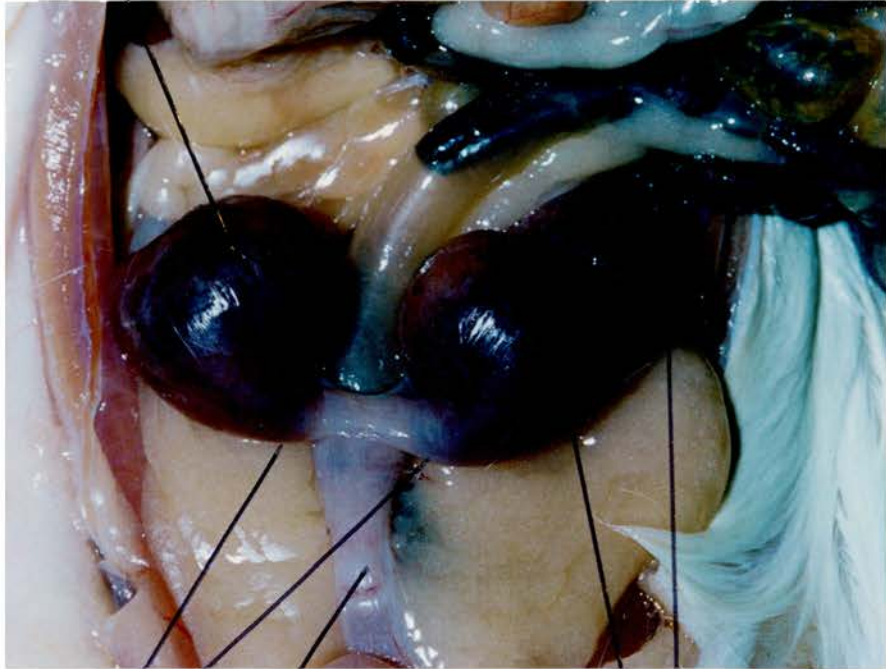
Endometrium, subplacenta, chorio-allantoic placenta, chorion and amnion from each uterine horn kept in the appropriate Tris buffer, and cut into 1-2 mm<sup>3</sup> sections.



Microsomal pellet prepared from each tissue type from one uterine horn, as described in section 2:10 and appropriate assay carried out.

**Figure 25b.** General procedure for preparing day 29 and day 36 pregnant guinea-pig fetal and uterine tissue for the detection of ACS, ACLS and PLA<sub>2</sub> enzyme activity.

Single conceptus

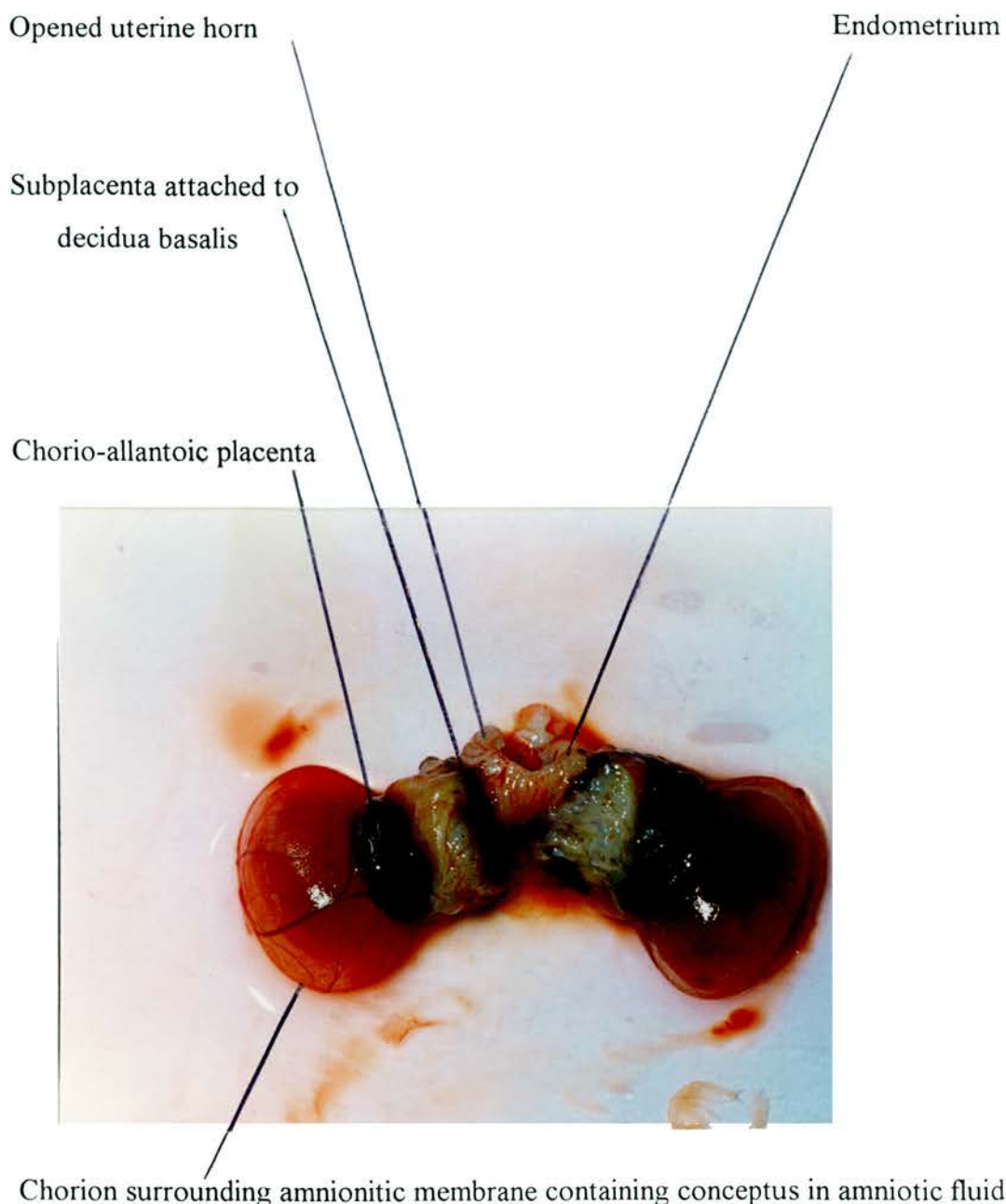


Uterine horns

Two conceptuses present  
in right uterine horn

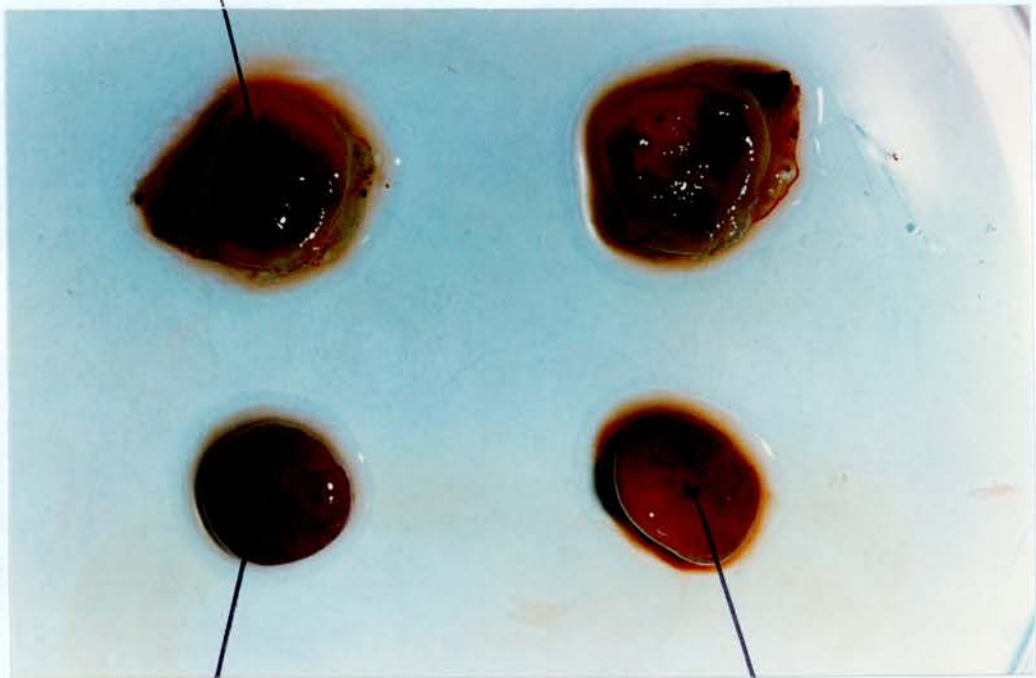
Cervix

**Figure 25c.** Photograph of ventral dissection of day 36 pregnant guinea-pig demonstrating position of cervix, uterine horns and conceptuses.



**Figure 25d.** Photograph demonstrating dissection of day 36 pregnant guinea-pig with single uterine horn opened, revealing two conceptuses attached to endometrium by subplacenta.

Subplacenta - note central mesenchyme  
where subplacenta is attached  
to chorio-allantoic placenta



Chorio-allantoic placenta

Position of  
umbilical cord  
in placenta

**Figure 25e.** Photograph of dissection of day 36 pregnant guinea-pig. The two placentae, namely the subplacenta and chorio-allantoic placenta, are shown.

pellet was prepared from each tissue and ACS, ACLS and PLA<sub>2</sub> assays were carried out as described in experiment 3:1.

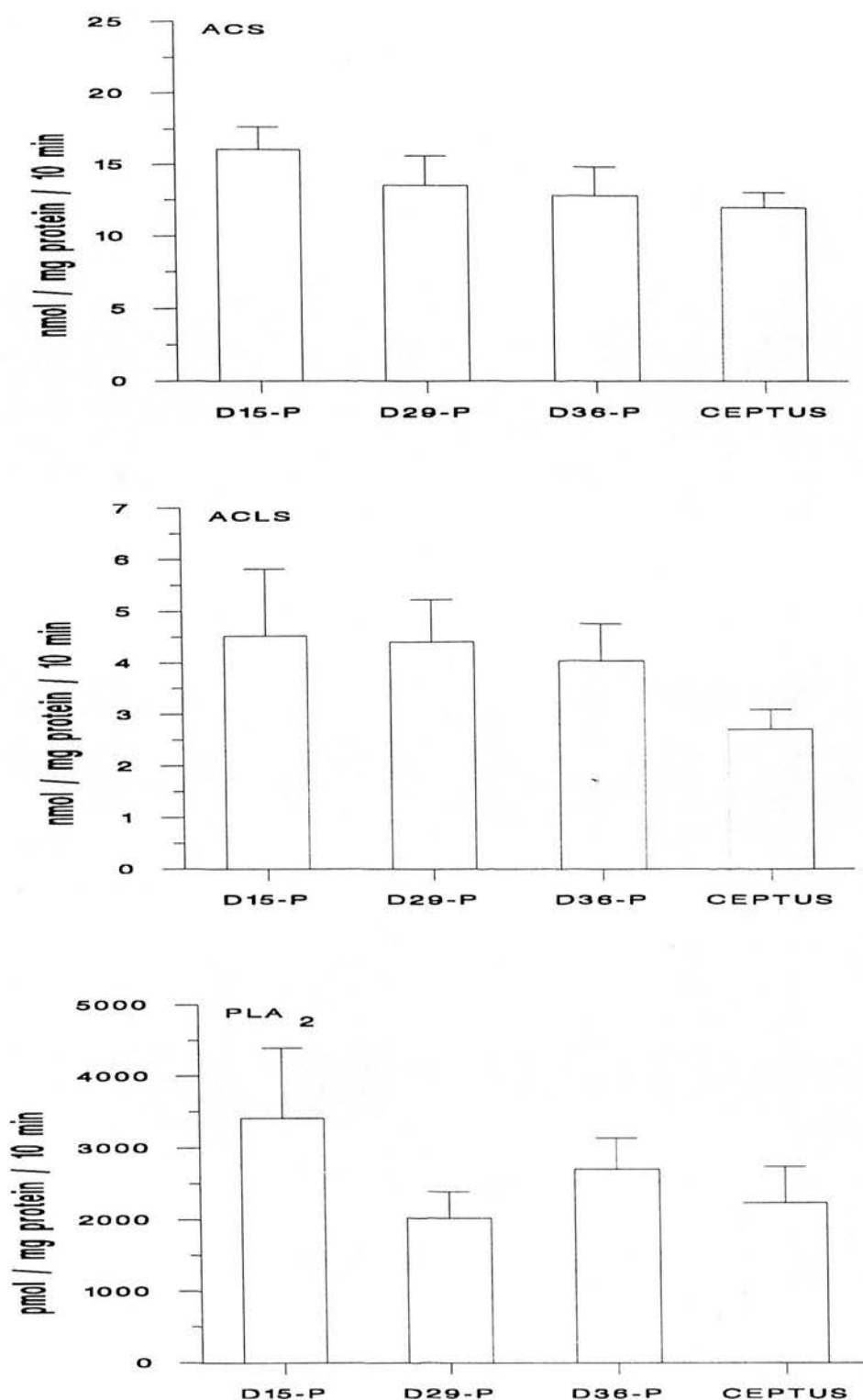
## Results

Detected ACS, ACLS and PLA<sub>2</sub> activities in the endometrium of day 15, 29 and 36 pregnant guinea-pigs are shown in Figure 26. Detected ACS, ACLS and PLA<sub>2</sub> activities in tissue from the conceptus of day 15 pregnant animals are also illustrated in Figure 26. Figure 27 illustrates detected ACS, ACLS and PLA<sub>2</sub> activities in tissue from the endometrium, subplacenta, chorio-allantoic placenta, chorion and amnion of day 29 and 36 pregnant guinea-pigs. All three enzymes were detected in all tissue types on all days of pregnancy.

## Discussion

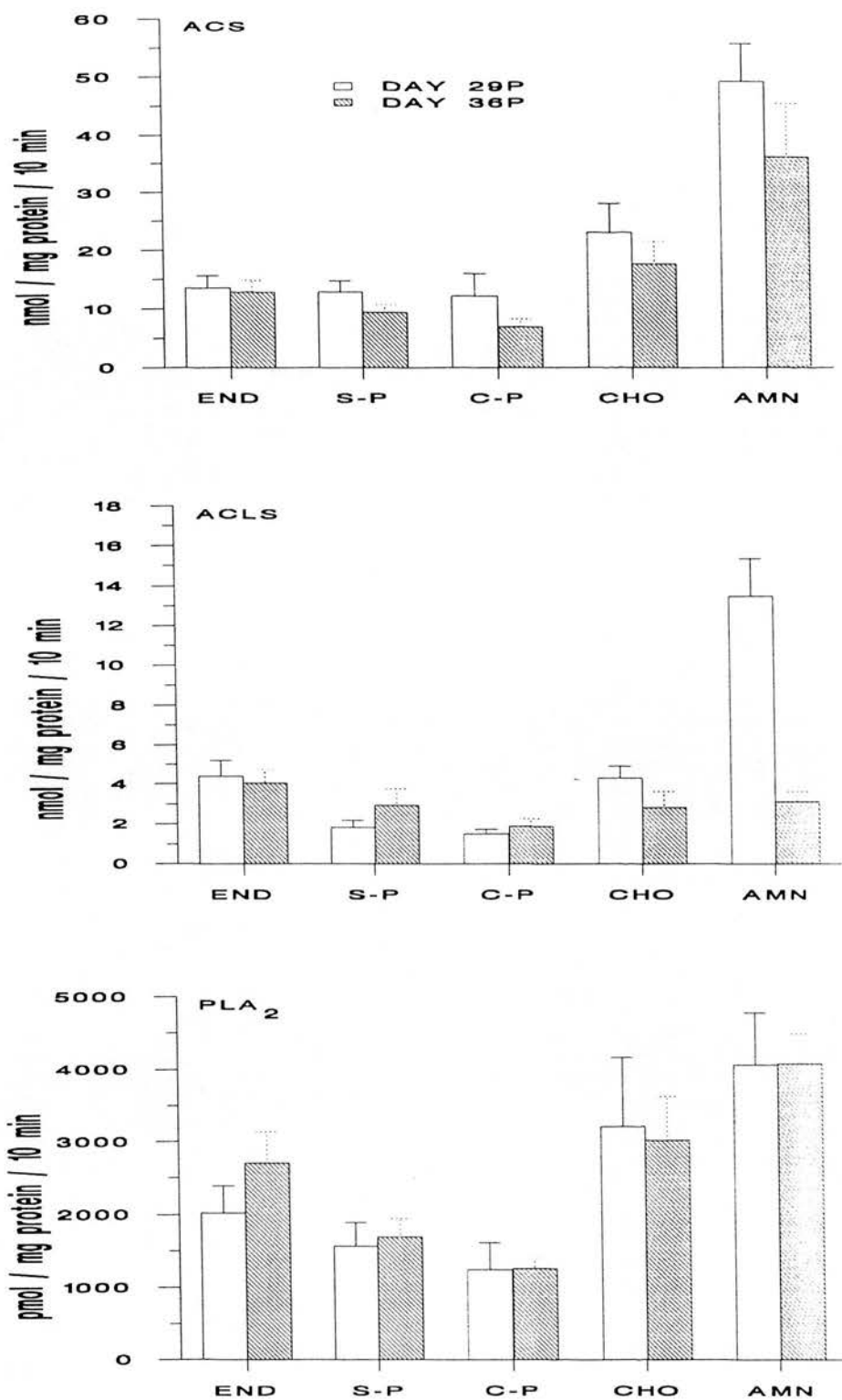
Due to the limitations of the assay methods (discussed in section 2:16), it was not possible to measure changes in the activities of ACS, ACLS and PLA<sub>2</sub> in the different tissues, or on different days of pregnancy. However, since ACS, ACLS and PLA<sub>2</sub> activities were all detected to be present in the uterine and conceptual tissues of day 15, 29 and 36 pregnant guinea-pigs, it is possible that these enzymes may have a role in the regulation of arachidonic acid turnover, and therefore prostaglandin synthesis, during pregnancy in the guinea-pig.

In order to fully understand the role of arachidonic acid turnover in relation to uterine PG production, both in non-pregnant and pregnant animals, it is necessary to investigate the actual quantities of PGs produced by uterine and conceptual tissues. Manipulation of the control of arachidonic acid turnover by inhibition of ACS, ACLS and PLA<sub>2</sub>, and the subsequent changes in PG production, have therefore been investigated.



**Figure 26.** Mean ( $\pm$  s.e.m.,  $n=5$ ) detected activities of ACS, ACLS and PLA<sub>2</sub> in the endometrium of day 15 pregnant (D15-P), day 29 pregnant (D29-P), day 36 pregnant (D36-P), and in the conceptus from day 15 pregnant (CEPTUS) guinea-pigs.





**Figure 27.** Mean ( $\pm$  s.e.m.,  $n=5$ ) detected activities of ACS, ACLS and PLA<sub>2</sub> in the endometrium (END), subplacenta (S-P), chorio-allantoic placenta (C-P), chorion (CHO), and amnion (AMN) in day 29 and 36 pregnant guinea-pigs.

**INVESTIGATIONS INTO THE INVOLVEMENT OF ACYL-CoA  
SYNTHETASE, ACYL-CoA:LYSOPHOSPHOLIPID ACYLTRANSFERASE  
AND PHOSPHOLIPASE A<sub>2</sub> ACTIVITIES IN THE REGULATION OF  
PROSTAGLANDIN RELEASE FROM THE GUINEA-PIG UTERUS.**

**3:4    Effects of inhibitors of arachidonic acid turnover on prostaglandin  
synthesis by day 7 and day 15 guinea-pig endometrium in culture.**

**Introduction**

ACS, ACLS and PLA<sub>2</sub> activity have been shown to be present on days 7 and 15 of the oestrous cycle (experiment 3:1) and in the presence of steroid hormones in ovariectomized animals (experiment 3:2). The following experiment has been carried out to investigate the role of arachidonic acid turnover in relation to actual PG synthesis in the uterus.

p-Hydroxymercuribenzoic acid (HMB) has been previously shown to inhibit both ACS and ACLS (also known as lysolecithin acyltransferase) at a concentration of 100 µM in the subcellular fraction of human lung fibroblasts (Hunter *et al.*, 1984). Inhibition of ACS by HMB led to decreased [<sup>14</sup>C]arachidonyl-CoA formation and also reduced the concentration of [<sup>14</sup>C]phosphatidylcholine generated as a result of inactivation of ACLS. It was concluded that this nonspecific inhibitor of many sulphhydryl enzymes (it is a sulphhydryl binding agent) could block the re-uptake of free arachidonic acid into lipid pools (Hunter *et al.*, 1984). Decreased incorporation into lipid pools following inhibition was unlikely to be attributed to the toxic effects of HMB, since greater than 90% of fibroblasts remained viable at the end of incubations and the fibroblasts were still capable of synthesizing arachidonic metabolites. This also suggests that HMB was probably without effect on PLA<sub>2</sub>, and since it has been indicated that PLA<sub>2</sub> does not contain sulphhydryl groups, HMB would be unable to form mercaptide bonds with the acylhydrolases (Hunter *et al.*, 1984). 150 µM HMB resulted in a large, dose-dependent stimulation of PGE<sub>2</sub> synthesis of 1490%, implicating the pathway of free arachidonic acid reacylation as a possible mechanism for the control of synthesis of arachidonic acid metabolites (Hunter *et al.*, 1984), such

as PGs. In rat peritoneal macrophages thimerosal (THM), (ethyl[2-mercaptobenzoato-S]mercury sodium salt, also known as merthiolate), treatment resulted in a large increase in PGE<sub>2</sub>, PGI<sub>2</sub> and TXB<sub>2</sub> synthesis (Goppelt-Struebe *et al.*, 1986). THM was demonstrated to be a rather selective inhibitor of ACLS, whilst PLA<sub>2</sub> remained unaffected, and dose-dependently inhibited the incorporation of free arachidonic acid into cellular phospholipids (at lower concentrations, specifically into PC) (Goppelt-Struebe *et al.*, 1986). The incorporation of arachidonic acid into PI was less sensitive towards THM than the incorporation into other lipids, suggesting that THM did not unspecifically inhibit the reacylation but rather specifically inhibited the ACLS (Goppelt-Struebe *et al.*, 1986). Unfortunately, this study could not investigate the effect of THM on ACS (the first enzyme in the reacylation pathway) since one of the substrates is CoA which would react with THM immediately. Therefore, the evidence for the specificity of action of THM remains indirect. In human platelets, THM treatment resulted in a 35-fold increase in thromboxane release. In rat peritoneal macrophages, high concentrations of exogenous free arachidonic acid resulted in greater quantities of prostanoids synthesized than could be achieved by THM treatment ie. when only endogenous arachidonic acid was available. The quantities of synthesized prostanoids in the presence of optimal concentrations of THM could be further enhanced by the addition of exogenous arachidonic acid. This would suggest that THM increased prostanoid synthesis by increasing the intracellular pool of precursor arachidonic acid, without activating the biosynthetic pathway (Goppelt-Struebe *et al.*, 1986). Sulphydryl reagents, such as HMB and THM, are usually mercury-containing compounds which readily react with sulphydryl (-SH) groups. Coenzyme A (CoA) contains a sulphydryl group and therefore, HMB and THM inhibit arachidonic acid turnover by reacting with CoA and arachidonyl-CoA, blocking ACS and ACLS activity.

Aristolochic acid (3,4-methylenedioxy-8-methoxy-10-nitro-1-phenanthrenecarboxylic acid), a nitrophenanthrene derivative isolated from *Aristolochia* species, is an herbal medicine used to neutralize snake venom toxicity. It binds directly (but not covalently) to *Vipera russelli* PLA<sub>2</sub> at domains other than the active site altering the  $\alpha$ -helical content of the protein (which results in an alteration of

the secondary structure of the molecule) thus inhibiting *in vitro* enzymatic activity (see Rosenthal, Vishwanath and Franson, 1989; Rosenthal, Lattanzio and Franson, 1992). Aristolochic acid has been demonstrated to inhibit various calcium-dependent PLA<sub>2</sub>s, such as those from human synovial fluid, neutrophils and platelets, as well as snake venom (see Rosenthal *et al.*, 1992). Calcium ionophore A23187-stimulated mobilization of [<sup>3</sup>H]-arachidonate was effectively antagonised by the protein-targeted PLA<sub>2</sub> inhibitor, aristolochic acid, in human neutrophils (Rosenthal *et al.*, 1992). PLA<sub>2</sub> activity is not only a function of substrate concentration and pH, but is greatly influenced by the physiochemical state of the phospholipid substrate. PLA<sub>2</sub> is essentially inactive against monomeric substrate whereas enzyme activity is greatly increased at concentrations equal or above the critical micelle concentration. Even with concentrations far exceeding the critical micelle concentration, any perturbation of the lipid-water interface diminishes enzyme activity. Complex formation between phospholipid substrate and drug also prevents enzyme attack of the substrate. Quinacrine (also known as mepacrine) has been shown to inhibit PLA<sub>2</sub> activity and does so by interfering with the substrate-enzyme interface and via complex formation (Chang, Musser and McGregor, 1987). However, under the strictest terms, it is inaccurate to consider quinacrine as a direct enzyme inhibitor, and conclusions drawn from its use in physiological experiments should be considered cautiously (Chang *et al.*, 1987). Quinacrine has also previously been shown to inhibit PGH synthase (Flower and Blackwell, 1976) and so the effect of this compound on PG production should be treated with some caution, since quinacrine may not only influence PG production by inhibiting PLA<sub>2</sub>, but also by inhibiting PGH synthase. The potential effects of all the above inhibitors on PG metabolism should also be considered. If any of these compounds were also able to inhibit the activity of PGDH, this may result in increased PG production, since PG metabolism would be reduced. The potential effects of the inhibitors on the activity of PGDH are unknown.

The effects of p-hydroxymercuribenzoic acid, thimerosal (THM) (ACS and ACLS inhibitors), aristolochic acid (ARA) and quinacrine (QUIN) (PLA<sub>2</sub> inhibitors) on prostaglandin synthesis by cultured day 7 and 15 guinea-pig endometrium have been studied.

**Methods**

The uteri were removed from five day 7 and five day 15 virgin guinea-pigs that had each displayed at least 3 normal oestrous cycles. Under aseptic conditions, the two uterine horns from each guinea-pig were “opened” by a longitudinal incision and the endometrium was dissected away from the myometrium. The endometrium was cut into 1-2 mm<sup>3</sup> pieces and approximately 15 and 40 mg wet weight (5-15 mg dry weight) of endometrium were placed in each Petri dish for culturing as explained in section 2:6. Sixteen dishes containing endometrium from each uterus were prepared with eight dishes being cultured in each of two modified Kilner jars. Each Kilner jar contained two control dishes (no treatment) which were regularly the top and bottom dishes to allow for any variation in culturing which may occur in the jars. Three concentrations of two inhibitor compounds were placed in the remaining six dishes per jar. The four inhibitors tested were p-hydroxymercuribenzoic acid (HMB) and thimerosal (THM) at 20, 100 and 500 µM, and aristolochic acid (ARA) and quinacrine (QUIN) at 10, 50 and 100 µM. The general set up (per animal) is shown below:-

<b>Kilner jar 1</b>	<b>Kilner jar 2</b>
(8 dishes)	(8 dishes)
Dish 1-Control	Dish 9-Control
Dish 2-HMB (20 µM)	Dish 10-ARA (10 µM)
Dish 3-HMB (100 µM)	Dish 11-ARA (50 µM)
Dish 4-HMB (500 µM)	Dish 12-ARA (100 µM)
Dish 5-THM (20 µM)	Dish 13-QUIN (10 µM)
Dish 6-THM (100 µM)	Dish 14-QUIN (50 µM)
Dish 7-THM (500 µM)	Dish 15-QUIN (100 µM)
Dish 8-Control	Dish 16-Control

The Petri dishes were incubated at 37°C for 24 h, and the culture medium was removed and replaced with fresh medium containing the same treatments every 6 h. The samples of culture medium obtained were stored at -20°C before being assayed



for  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  by radioimmunoassay (as described in section 2:5). After culture, the pieces of endometrium were removed from each Petri dish into preweighed containers and dried by placing in an oven at  $37^\circ\text{C}$  for 24 h. Each container was then reweighed and the amount of dry endometrium from each Petri dish was calculated. The outputs of prostaglandins were calculated per mg dry weight of endometrium per 6 h of culture.

## Statistical Tests

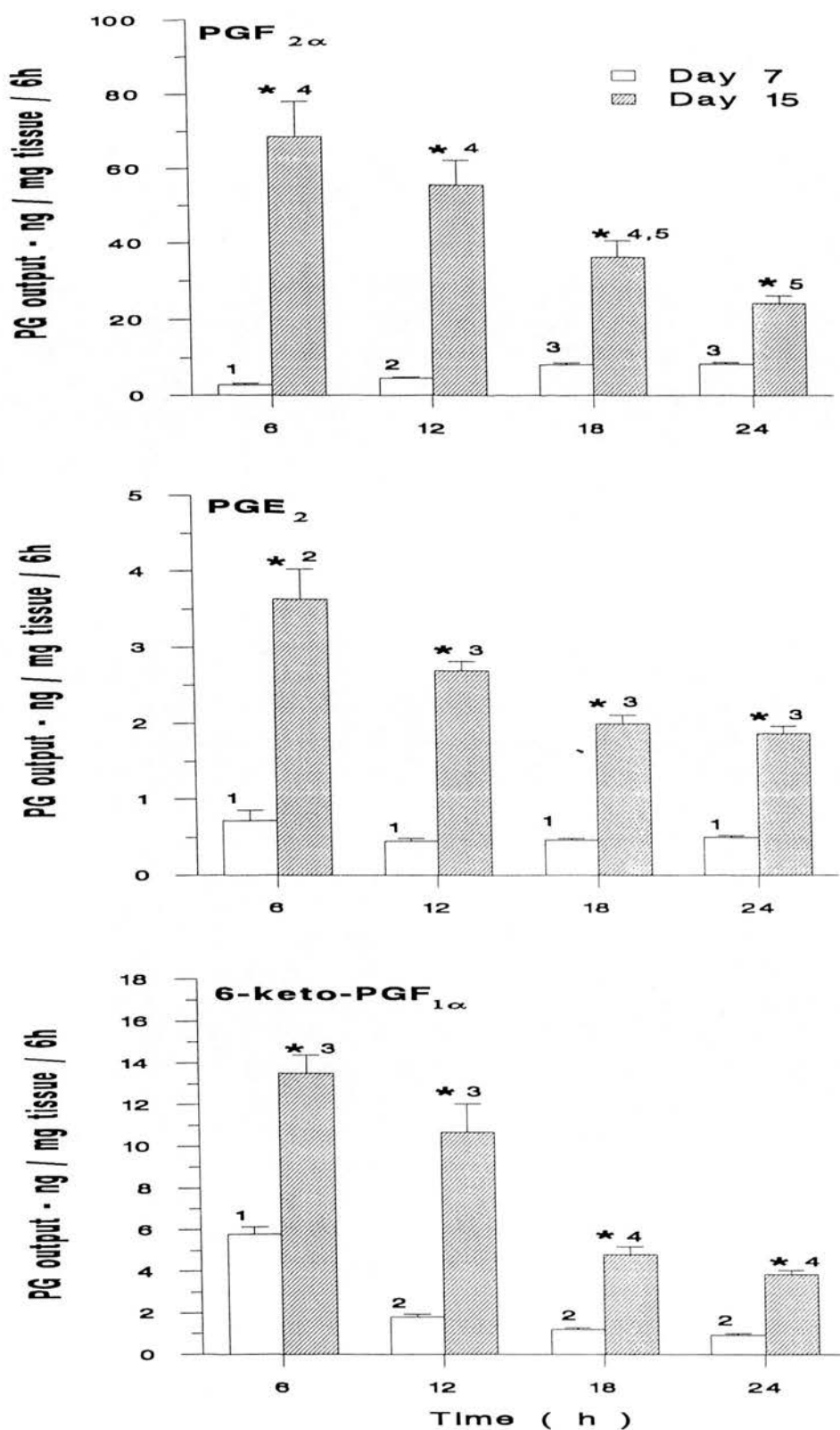
Changes in the output of PGs with time were analysed by Duncan's multiple range test. Differences between treated and control groups were analyzed by Student's t-test or, if the variances of the two groups were significantly different by the variance ratio F test, by a modified t-test for unequal variances.

## Results

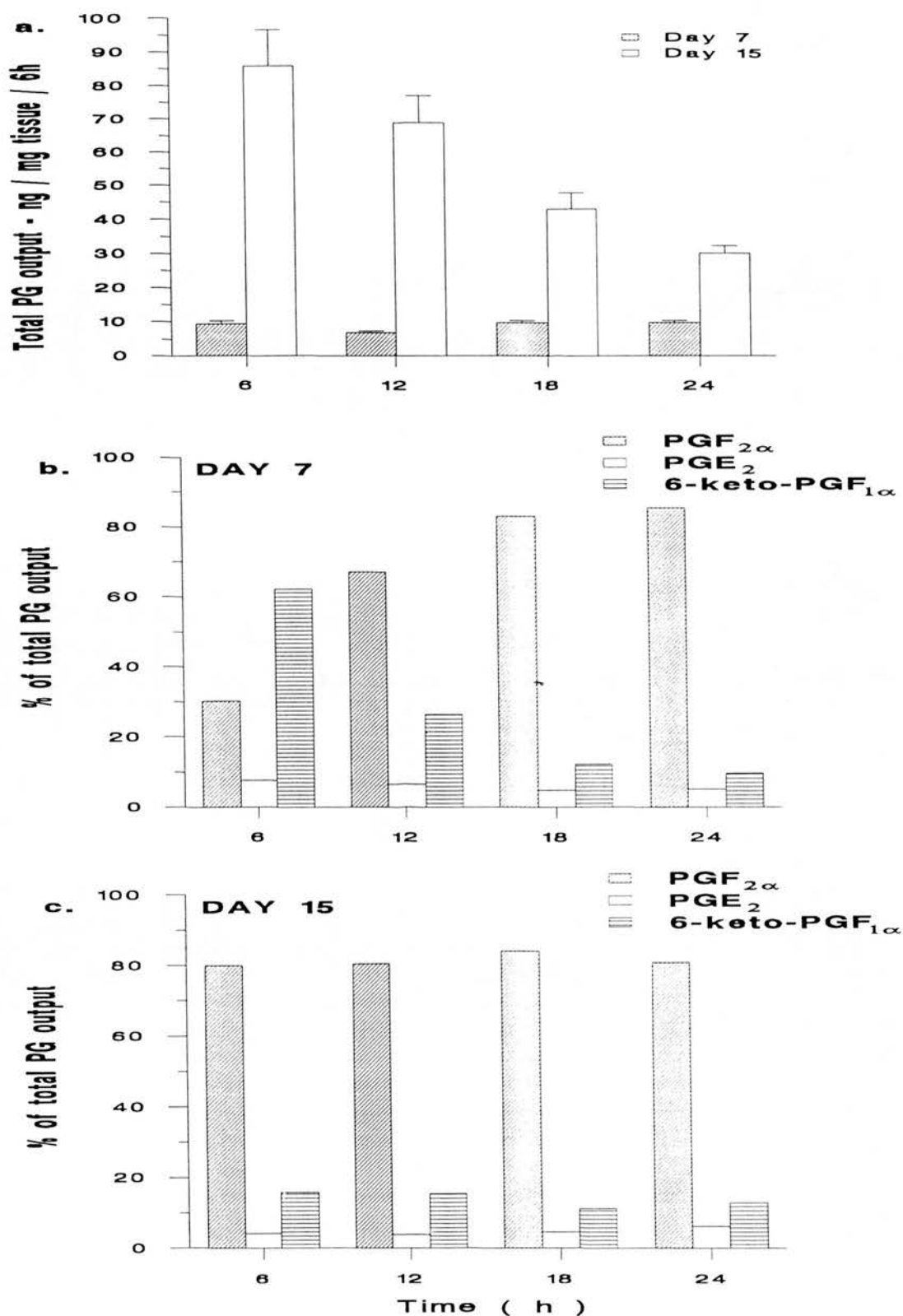
### Basal PG outputs

The basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 and day 15 guinea-pig endometrium cultured for 24 h and sampled at 6 h intervals are shown in Figure 28. The outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 endometrium and of 6-keto- $\text{PGF}_{1\alpha}$  from day 7 endometrium significantly ( $P < 0.05$ ) decreased with time. During culture of day 7 endometrium,  $\text{PGF}_{2\alpha}$  output significantly ( $P < 0.05$ ) increased with time, whilst  $\text{PGE}_2$  output remained unchanged. Figure 29 illustrates the total PG output of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 and day 15 guinea-pig endometrium during 24 h culture, and the percentage of each PG produced.  $\text{PGF}_{2\alpha}$  was the major PG released from day 15 endometrium (approximately 80 % of total PG output), with much lower quantities of 6-keto- $\text{PGF}_{1\alpha}$  and  $\text{PGE}_2$  (approximately 15 and 5 %, respectively). Initially, 6-keto- $\text{PGF}_{1\alpha}$  was the major PG released from day 7 endometrium (approximately 60 % of total PG output), with lower amounts of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  (approximately 30 and 10 % of total PG output). However, as 6-keto- $\text{PGF}_{1\alpha}$  output declined and  $\text{PGF}_{2\alpha}$  output increased,  $\text{PGF}_{2\alpha}$  was the major PG released from day 7 endometrium after 12, 18 and 24 h. The outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-





**Figure 28.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and  $6\text{-keto-PGF}_{1\alpha}$  from day 7 and day 15 non-pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h. Columns with the same number for one particular PG on one particular day are not significantly ( $P < 0.05$ ) different. \* Significantly ( $P < 0.05$ ) higher than day 7 value.



**Figure 29.** Total PG output from day 7 and day 15 endometrium cultured for 24 h and sampled every 6 h (Fig. a.), and the percentage PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs of the total PG output in day 7 (Fig. b.) and day 15 (Fig. c.) non-pregnant guinea-pig endometrium.

keto-PGF<sub>1α</sub> were significantly ( $P < 0.05$ ) greater from day 15 endometrium than from day 7 endometrium at all times (see Figure 28).

#### Effects of HMB, THM, ARA and QUIN treatments on PG output

i. Day 7 endometrium - The effects of HMB, THM, ARA and QUIN treatments on PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs from day 7 guinea-pig endometrium cultured for 24 h and sampled at 6 h intervals are shown in Figures 30, 31, 32 and 33, respectively. HMB, THM, ARA and QUIN had no effect on the outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 7 endometrium during the first 6 h of culture.

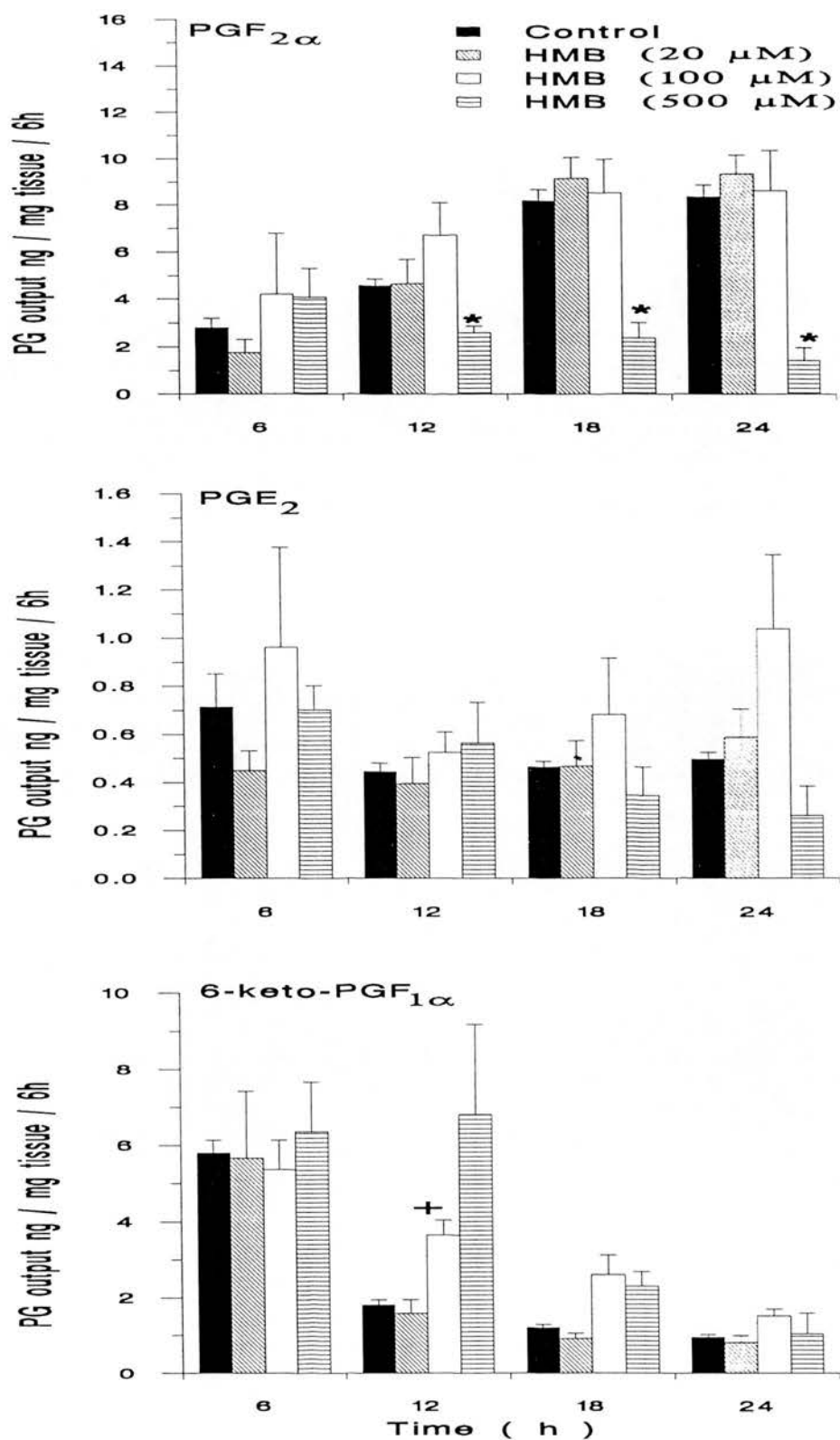
*PGF<sub>2α</sub> output* - HMB (500 μM), THM (100 and 500 μM) and ARA (10, 50 and 100 μM) significantly ( $P < 0.05$ ) decreased PGF<sub>2α</sub> output at 12, 18 and 24 h of culture. 20 μM THM also significantly ( $P < 0.05$ ) decreased PGF<sub>2α</sub> output at 18 and 24 h. 100 μM QUIN significantly ( $P < 0.05$ ) decreased PGF<sub>2α</sub> output at 12 and 18 h of culture.

*PGE<sub>2</sub> output* - None of the inhibitors caused any significant changes in PGE<sub>2</sub> output.

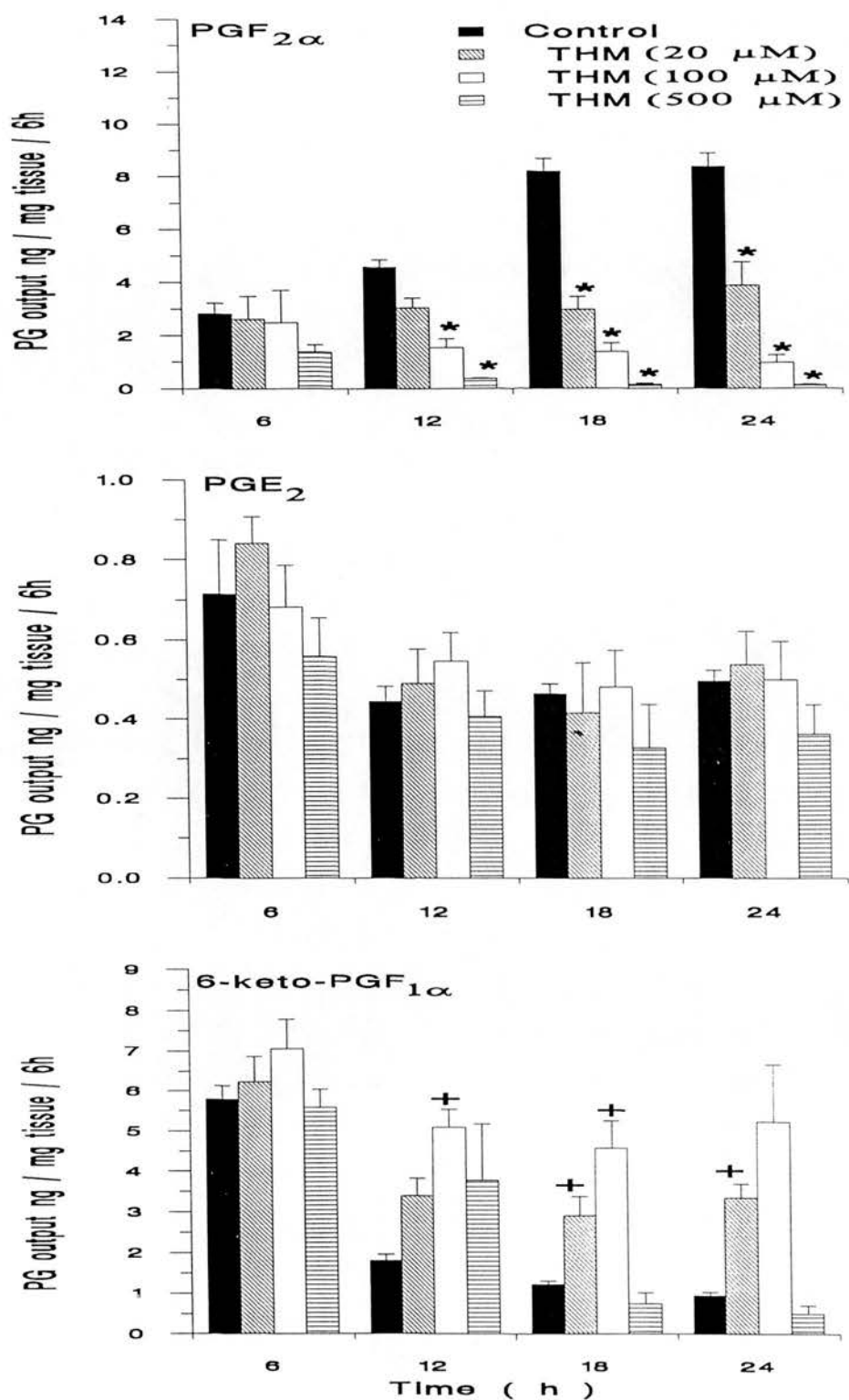
*6-keto-PGF<sub>1α</sub> output* - The output of 6-keto-PGF<sub>1α</sub> was significantly ( $P < 0.05$ ) increased by 100 μM HMB at 12 h, by 100 μM THM at 12 and 18 h, and by 20 μM THM at 18 and 24 h of culture. No significant changes in 6-keto-PGF<sub>1α</sub> output were observed with ARA and QUIN treatment, except for 50 μM ARA which caused a significant ( $P < 0.05$ ) decrease in 6-keto-PGF<sub>1α</sub> output at 18 h of culture.

ii. Day 15 endometrium - The effects of HMB, THM, ARA and QUIN treatments on PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs from day 15 guinea-pig endometrium cultured for 24 h and sampled at 6 h intervals are shown in Figures 34, 35, 36 and 37, respectively.

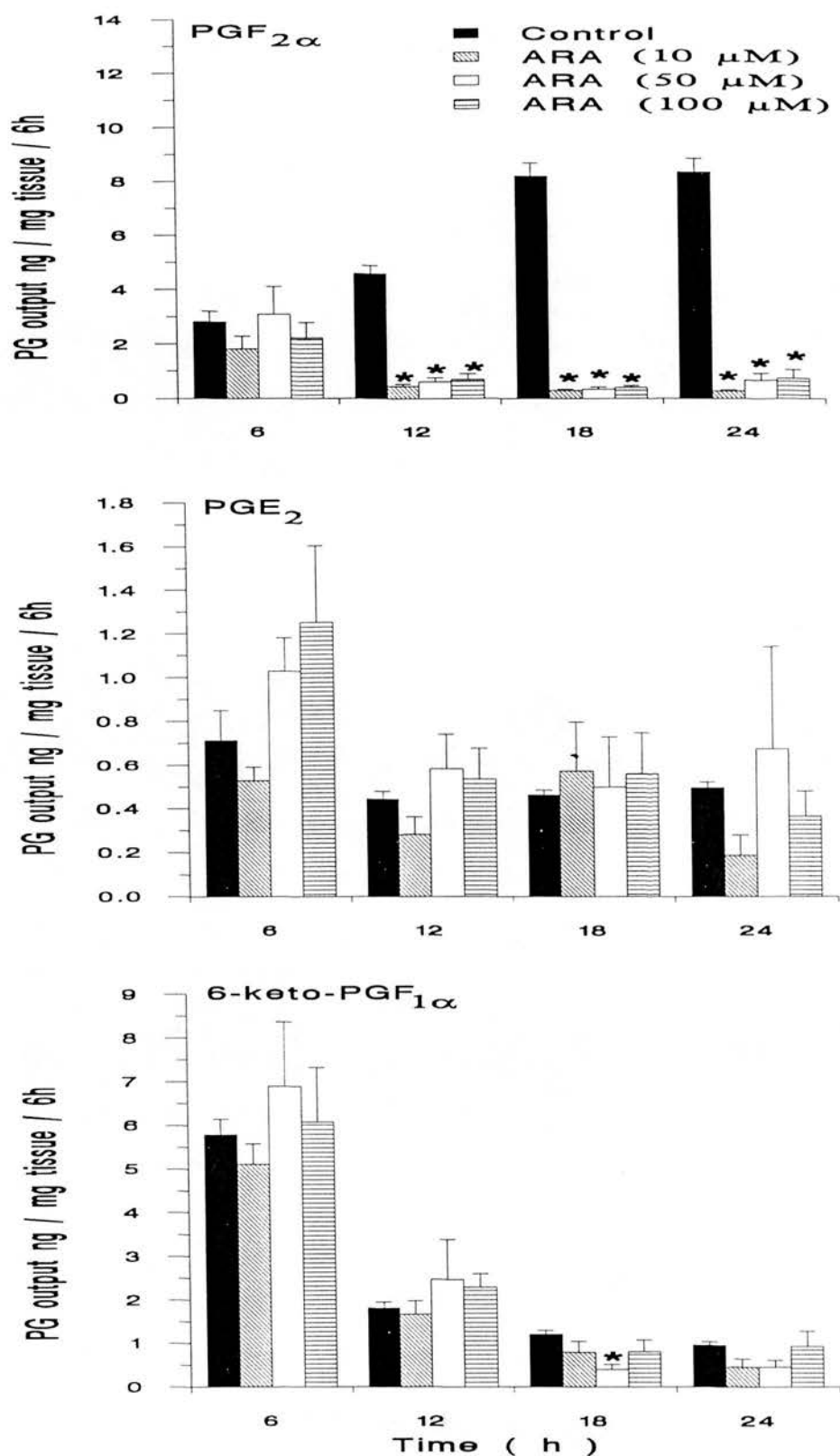
*PGF<sub>2α</sub> output* - PGF<sub>2α</sub> output was significantly ( $P < 0.05$ ) decreased by 500 μM HMB at 18 h, by 500 and 100 μM THM at 6, 12, 18 and 24 h, and by 20 μM THM at 12 and 18 h of culture. PGF<sub>2α</sub> output was significantly ( $P < 0.05$ ) decreased by 50 and 100 μM ARA at 12 h and by 10, 50 and 100 μM ARA at 6, 18 and 24 h of culture. QUIN also tended to decrease PGF<sub>2α</sub> output, but this was only significant at 6 h of culture with 50 μM QUIN ( $P < 0.05$ ). With regard to PGF<sub>2α</sub> output, THM and ARA appeared to act in a dose-dependent manner.



**Figure 30.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 7 non-pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of HMB (20, 100 and 500  $\mu\text{M}$ ). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.

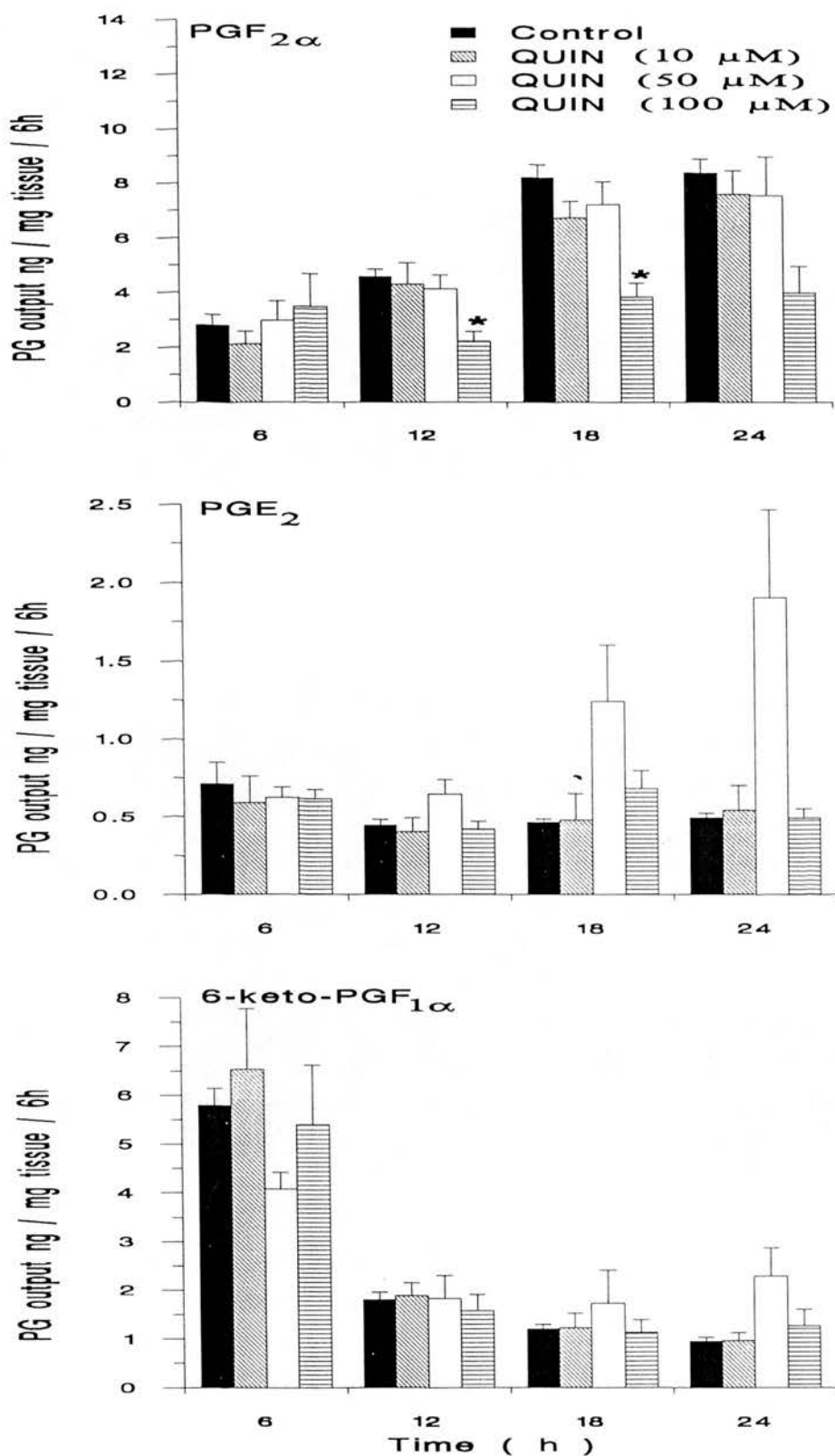


**Figure 31.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 7 non-pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500 μM). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.



**Figure 32.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 7 non-pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM). \* Significantly ( $P < 0.05$ ) lower than the control value for the same PG at the same time.





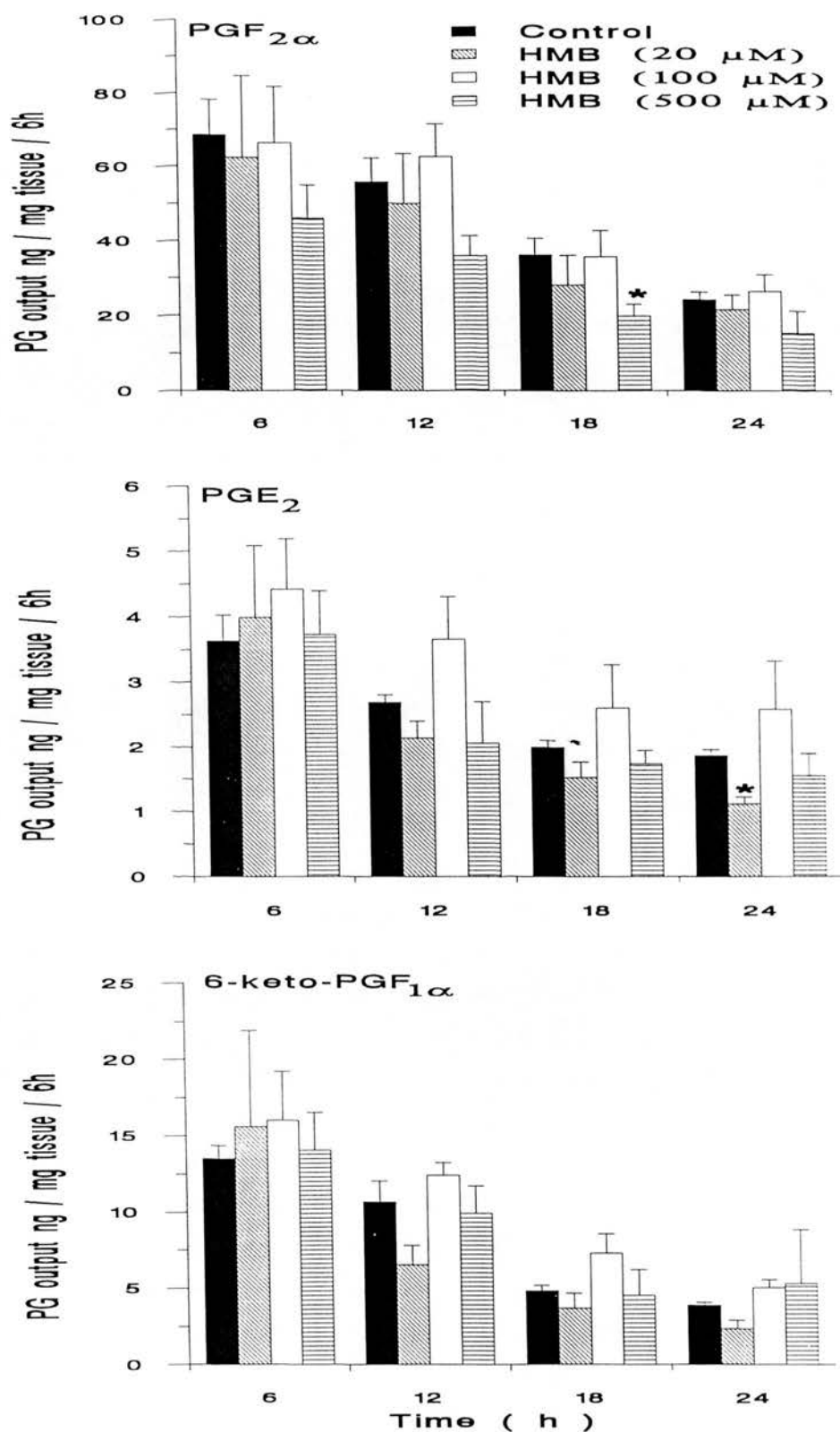
**Figure 33.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 7 non-pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of QUIN (10, 50 and 100 μM). \* Significantly ( $P < 0.05$ ) lower than the control value for the same PG at the same time.

*PGE<sub>2</sub> output* - PGE<sub>2</sub> output was significantly ( $P < 0.05$ ) decreased by 20  $\mu$ M HMB at 24 h, by 500  $\mu$ M THM at 6, 12, 18 and 24 h and by 100  $\mu$ M THM at 24 h of culture. 100  $\mu$ M ARA at 6, 12 and 18 h, and 10  $\mu$ M ARA at 18 h of culture, also significantly reduced PGE<sub>2</sub> output ( $P < 0.05$ ). PGE<sub>2</sub> output was significantly ( $P < 0.05$ ) decreased by 50  $\mu$ M QUIN at 6 and 12 h and by 100  $\mu$ M QUIN at 6 h of culture. However, at 24 h of culture, 50  $\mu$ M QUIN significantly ( $P < 0.05$ ) increased PGE<sub>2</sub> output.

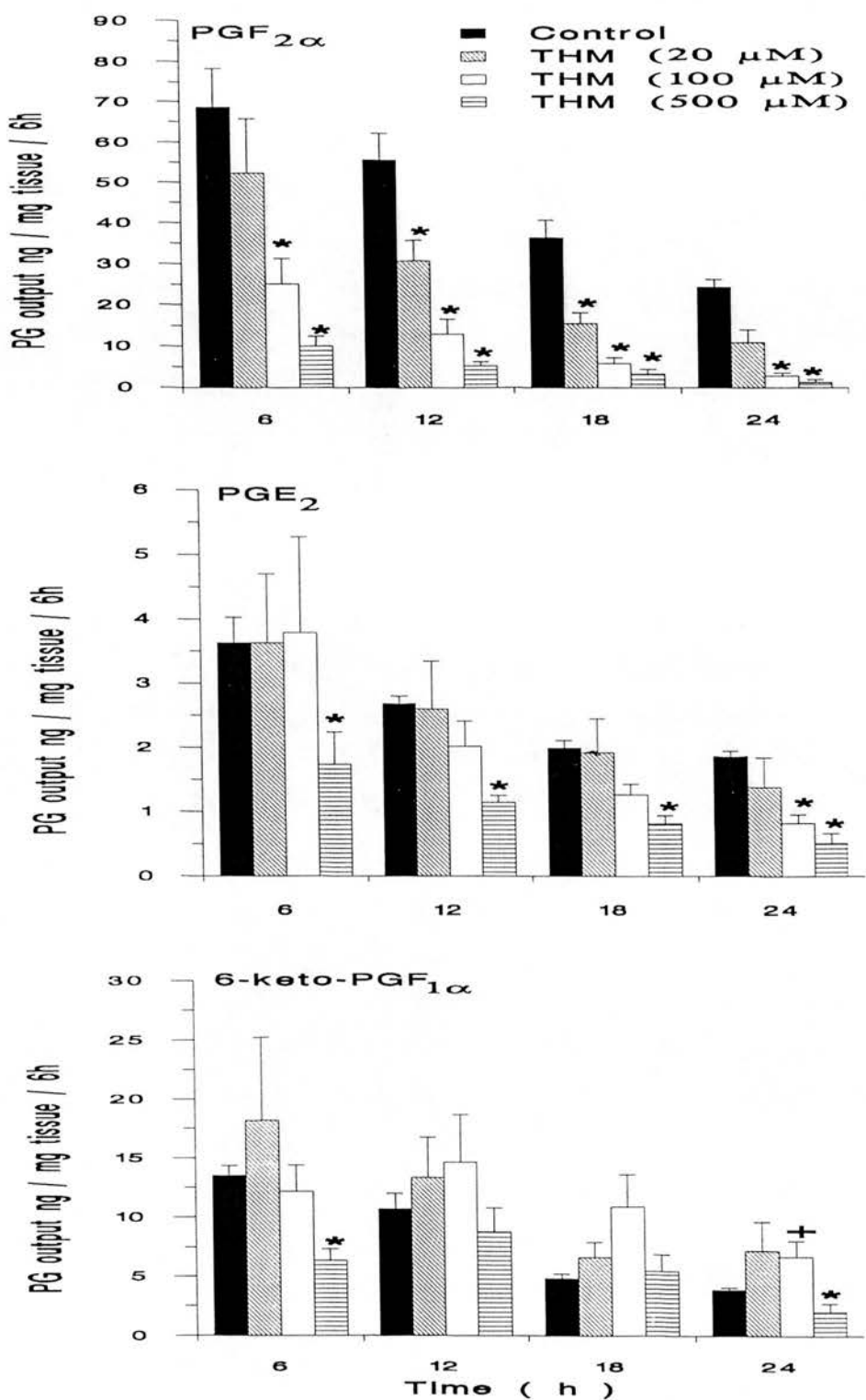
*6-keto-PGF<sub>1 $\alpha$</sub>  output* - HMB had no effect on 6-keto-PGF<sub>1 $\alpha$</sub>  output. 500  $\mu$ M THM significantly ( $P < 0.05$ ) decreased 6-keto-PGF<sub>1 $\alpha$</sub>  output at 6 and 12 h, whilst 100  $\mu$ M THM significantly ( $P < 0.05$ ) increased output at 24 h of culture. 6-keto-PGF<sub>1 $\alpha$</sub>  output was significantly ( $P < 0.05$ ) decreased by 10  $\mu$ M ARA at 6 h, by 50  $\mu$ M ARA at 12 and 18 h, and by 100  $\mu$ M ARA at 6 h of culture. 6-keto-PGF<sub>1 $\alpha$</sub>  output was significantly ( $P < 0.05$ ) decreased by 50  $\mu$ M QUIN at 12 h and significantly ( $P < 0.05$ ) increased by 100  $\mu$ M QUIN at 24 h of culture.

Table 7. Summary of the general effects of the four inhibitors on PG output from day 7 and day 15 endometrium during 24 h culture, where  $\uparrow$  and  $\downarrow$  represent an increase and decrease in PG output, respectively. The greater the number of arrows, the greater the influence of the inhibitors on PG output i.e.  $\uparrow\uparrow\uparrow$  represents a much greater increase in PG output than  $\uparrow$ . “Sig.” illustrates whether the change was significant.

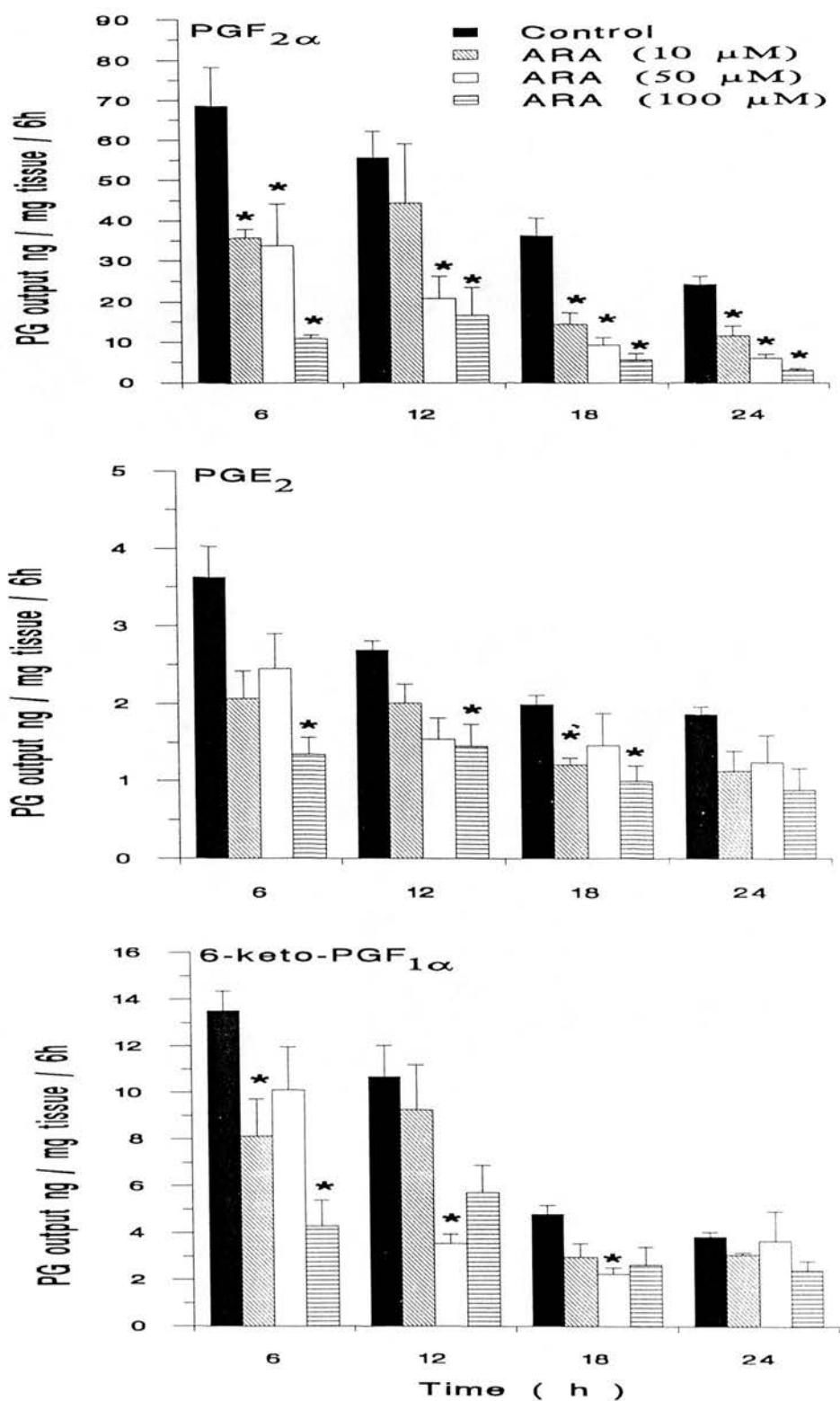
Treatment	<u>Day 7</u> PGF <sub>2<math>\alpha</math></sub>	<u>Day 7</u> PGE <sub>2</sub>	<u>Day 7</u> 6-keto- PGF <sub>1<math>\alpha</math></sub>	<u>Day 15</u> PGF <sub>2<math>\alpha</math></sub>	<u>Day 15</u> PGE <sub>2</sub>	<u>Day 15</u> 6-keto- PGF <sub>1<math>\alpha</math></sub>
HMB	$\downarrow\downarrow\downarrow$ sig.	No effect	$\uparrow$ sig.	$\downarrow$ sig.	$\downarrow$ sig.	No effect
THM	$\downarrow\downarrow\downarrow$ sig.	No effect	$\uparrow\uparrow$ sig.	$\downarrow\downarrow\downarrow$ sig.	$\downarrow\downarrow$ sig.	$\downarrow$ & $\uparrow$ sig.
ARA	$\downarrow\downarrow\downarrow$ sig.	No effect	$\downarrow$ sig.	$\downarrow\downarrow$ sig.	$\downarrow$ sig.	$\downarrow$ sig.
QUIN	$\downarrow$ sig.	No effect	No effect	$\downarrow$ sig.	$\downarrow$ & $\uparrow$ sig.	$\downarrow$ & $\uparrow$ sig.



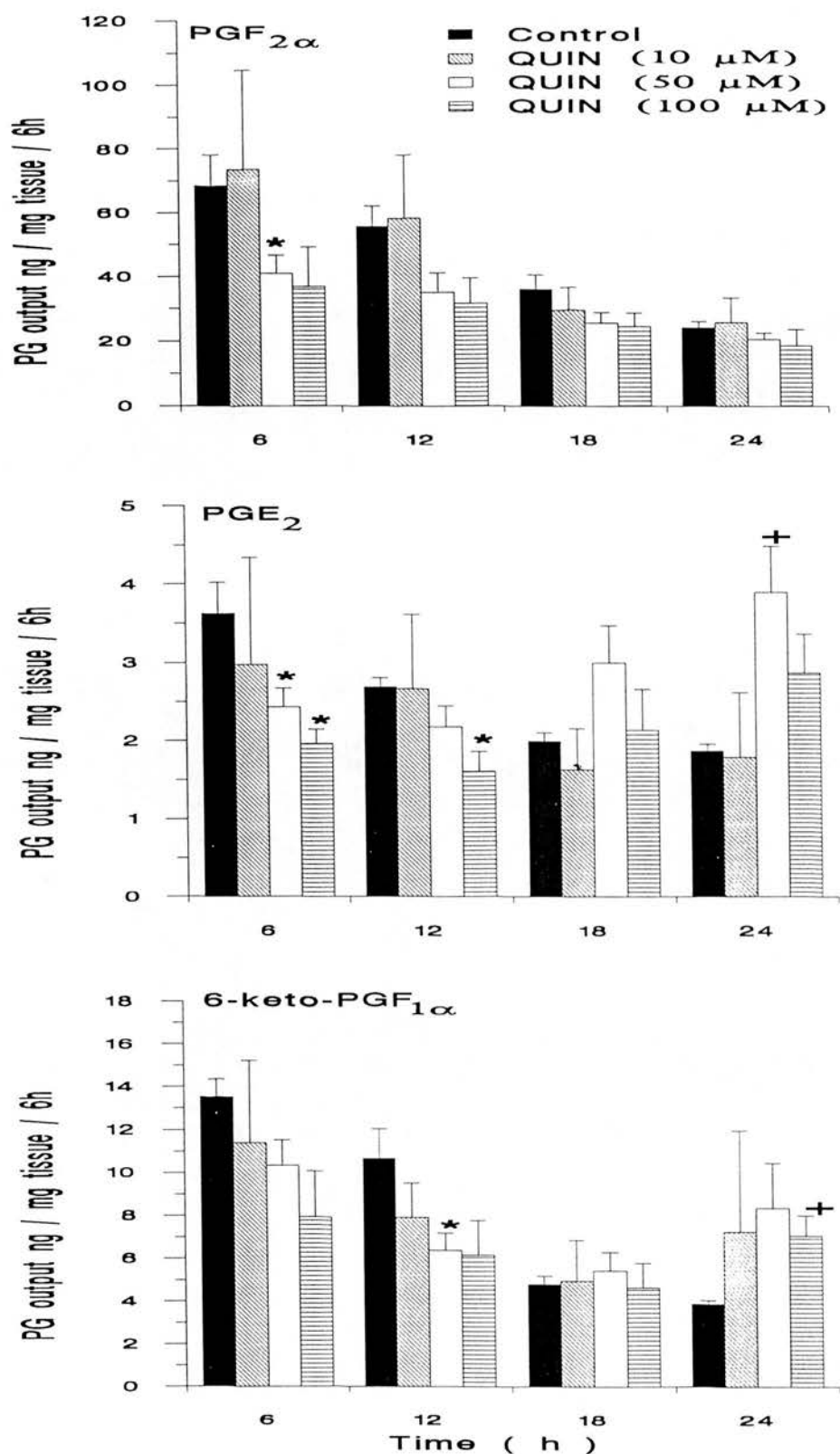
**Figure 34.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 15 non-pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of HMB (20, 100 and 500 μM). \* Significantly ( $P < 0.05$ ) lower than the control value for the same PG at the same time.



**Figure 35.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 non-pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500  $\mu\text{M}$ ). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.



**Figure 36.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 non-pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100  $\mu\text{M}$ ). \* Significantly ( $P < 0.05$ ) lower than the control value for the same PG at the same time.



**Figure 37.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 15 non-pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of QUIN (10, 50 and 100  $\mu\text{M}$ ). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.



## Discussion

By inhibiting arachidonic acid reacylation into lysophospholipids with HMB and THM (i.e. by inhibiting acyl-CoA synthetase and acyl-CoA:lysophospholipid acyltransferase activities), it was thought that an increase in free arachidonic acid would occur, thereby resulting in an increase in prostaglandin production by the guinea-pig endometrium in culture. However, HMB and THM treatment actually decreased  $\text{PGF}_{2\alpha}$  production from both day 7 and day 15 endometrium. In contrast to  $\text{PGF}_{2\alpha}$ , 6-keto- $\text{PGF}_{1\alpha}$  output from day 7 endometrium was significantly increased by HMB and THM treatment, suggesting that  $\text{PGF}_{2\alpha}$  and  $\text{PGI}_2$  (as detected by 6-keto- $\text{PGF}_{1\alpha}$ ) syntheses are controlled differently.

The effects of HMB and THM treatment on prostaglandin production were found to be different in day 15 animals.  $\text{PGF}_{2\alpha}$  output was still decreased by the inhibitors, however  $\text{PGE}_2$  output was also significantly decreased. The output of 6-keto- $\text{PGF}_{1\alpha}$  was unaffected by HMB, and was both increased and decreased by THM treatment. The lack of effect of the inhibitors on  $\text{PGE}_2$  output from day 7 endometrium may be due to the very low quantities of the prostaglandin produced at that time of the cycle (approximately 0.5 ng / mg tissue / 6 h). This would explain why the inhibitors affected  $\text{PGE}_2$  output from day 15 (when greater quantities of prostaglandin are produced) and not from day 7 endometrium.

ARA, and to a lesser extent QUIN, significantly inhibited  $\text{PGF}_{2\alpha}$  production from both day 7 and day 15 endometrium, thus  $\text{PLA}_2$  appears to have a crucial role in the regulation of arachidonic acid release for endometrial  $\text{PGF}_{2\alpha}$  synthesis in the guinea-pig. The inhibition of  $\text{PLA}_2$  had no effect on  $\text{PGE}_2$  output from day 7 animals, but did reduce  $\text{PGE}_2$  output from day 15 endometrium. Similarly to the results observed following HMB and THM treatment, the lack of effect of ARA and QUIN on  $\text{PGE}_2$  output from day 7 animals may be due to the very low concentrations of  $\text{PGE}_2$  produced at that time. ARA treatment significantly reduced 6-keto- $\text{PGF}_{1\alpha}$  output from both day 7 and day 15 endometrium. However, the reduction in prostaglandin output was not as striking as that seen with  $\text{PGF}_{2\alpha}$  output. QUIN had very little effect on 6-keto- $\text{PGF}_{1\alpha}$  output.

The results with ARA and QUIN clearly indicate the vital role PLA<sub>2</sub> plays in the control of prostaglandin production, particularly of PGF<sub>2α</sub>, from the guinea-pig endometrium. The role of the reacylation enzymes, ACS and ACLS, is not as well defined. The use of inhibitors which supposedly prevent the reacylation of arachidonic acid did not increase prostaglandin output from guinea-pig endometrium on either day 7 or day 15 of the oestrous cycle. Therefore ACS and ACLS do not appear to play a significant role in the regulation of endometrial prostaglandin synthesis. However, inhibition of ACS and ACLS will not only inhibit the "mopping up" of free arachidonic acid but will also inhibit the turnover of arachidonic acid between membrane phospholipid stores, thus possibly limiting the amount of arachidonic acid available in phospholipid stores suitable for the action of PLA<sub>2</sub>. This may be particularly true during 24 h tissue culture, due to the long time period of the experiment, and may explain why ACS and ACLS inhibited PGF<sub>2α</sub> output.

Another point that should not be overlooked is the potential toxic effects of the inhibitors on the tissue. This may be of relevance when using high inhibitor concentrations and during long-term 24 h culture experiments, such as these. This may explain the decrease, rather than increase, in PG production following arachidonic acid uptake inhibition. However, if toxic effects were being felt, one may have expected all PG synthesis to decrease during the 24 h culture, but this was not the case.

In order to further investigate the role of arachidonic acid turnover in the guinea-pig uterus, the effects of arachidonic acid turnover inhibitors have been investigated in uterine and conceptual tissues of early- and mid-pregnant guinea-pigs during 24 h tissue culture. Only THM and ARA were used in the following experiment since these had been shown to be the most effective inhibitors in the previous experiment.

### **3:5     Effects of thimerosal and aristolochic acid on prostaglandin synthesis by day 22, 29 and 36 pregnant guinea-pig uterine and conceptual tissue in culture.**

#### **Introduction**

PGF<sub>2α</sub> output from the guinea-pig uterus increases from about day 11 of the oestrous cycle. This increase does not occur if the animals are pregnant. The results observed in section 3:3 suggested that the control of arachidonic acid turnover may have a role in conceptual tissues during early- and mid-pregnancy in the guinea-pig. Consequently, the prostaglandin output from endometrium, subplacenta, chorio-allantoic placenta and fetal membranes (chorion and amnion) of day 22, 29 and 36 pregnant guinea-pigs, and the effects of inhibitors of arachidonic acid turnover on PG output, during 24 h of tissue culture and sampled every 6 h have been investigated.

#### **Methods**

Fifteen virgin female guinea-pigs which had undergone at least 3 normal oestrous cycles were mated by placing them separately on days 14-15 of the cycle (several days prior to oestrus) with a male guinea-pig of proven fertility. The guinea-pigs were examined daily for signs of mating and for the presence of sperm in the vaginal smear, with day 1 of pregnancy being designated the first day of observing any of these signs. Five guinea-pigs were each killed by stunning and incision of the neck, on days 22, 29 and 36 of pregnancy. Under aseptic conditions the uterus was removed from each animal and separated into its two horns. Each uterine horn was "opened" by a longitudinal incision and the conceptuses, along with the placenta, were removed, by gently pulling them away from the endometrium. The subplacenta, chorio-allantoic placenta, chorion and amnion were then separated from each conceptus in exactly the same way as described in section 3:3. All tissues were kept ice-cold and cut into 1-2 mm<sup>3</sup> pieces. The endometrium was separated from the myometrium by cutting away 1-2 mm<sup>3</sup> sections of endometrium using a pair of fine scissors. Approximately 15-40 mg wet weight (5-15 mg dry weight) of each tissue type (endometrium, subplacenta,

chorio-allantoic placenta and fetal membranes) were placed in each Petri dish for culturing as explained in section 2:6.

Day 29 and 36 pregnant guinea-pigs

Thirty-two Petri dishes containing tissue from each guinea-pig were prepared with eight dishes being cultured in each of four modified Kilner jars. Each Kilner jar contained two control dishes (no treatment) which were regularly the top and bottom dishes to allow for any variation in culturing which may occur in the jars. Three concentrations of two inhibitor compounds were placed in the remaining six dishes per jar. The two inhibitors tested were thimerosal (THM) at 20, 100 and 500 µM and aristolochic acid (ARA) at 10, 50 and 100 µM. The general set up (per animal) is shown below:-

<u>Kilner jar 1</u>	<u>Kilner jar 2</u>	<u>Kilner jar 3</u>	<u>Kilner jar 4</u>
(8 dishes)	(8 dishes)	(8 dishes)	(8 dishes)
Endometrium	Subplacenta	Chorio-allantoic placenta	Fetal membranes
1-Control	9-Control	17-Control	25-Control
2-THM (20 µM)	10-THM	18-THM	26-THM
3-THM (100 µM)	11-THM	19-THM	27-THM
4-THM (500 µM)	12-THM	20-THM	28-THM
5-ARA (10 µM)	13-ARA	21-ARA	29-ARA
6-ARA (50 µM)	14-ARA	22-ARA	30-ARA
7-ARA (100 µM)	15-ARA	23-ARA	31-ARA
8-Control	16-Control	24-Control	32-Control

Day 22 pregnant guinea-pigs

Exactly the same procedure was used to set up uterine and conceptual tissue from day 22 pregnant animals as from day 29 and 36 pregnant guinea-pigs. However, in day 22 pregnant guinea-pigs, insufficient fetal membrane tissue was available to prepare all

eight Petri dishes for culture. Therefore, in day 22 pregnant animals, only two dishes were prepared for “Kilner jar 4”. The general set up (per animal) is shown below:-

<u>Kilner jar 1</u>	<u>Kilner jar 2</u>	<u>Kilner jar 3</u>	<u>Kilner jar 4</u>
(8 dishes)	(8 dishes)	(8 dishes)	(8 dishes)
Endometrium	Subplacenta	Chorio-allantoic placenta	Fetal membranes
1-Control	9-Control	17-Control	25-Control
2-THM (20 $\mu$ M)	10-THM	18-THM	None
3-THM (100 $\mu$ M)	11-THM	19-THM	None
4-THM (500 $\mu$ M)	12-THM	20-THM	None
5-ARA (10 $\mu$ M)	13-ARA	21-ARA	None
6-ARA (50 $\mu$ M)	14-ARA	22-ARA	None
7-ARA (100 $\mu$ M)	15-ARA	23-ARA	None
8-Control	16-Control	24-Control	26-Control

All Petri dishes were incubated at 37°C for 24 h, and the culture medium was removed and replaced with fresh medium containing the same treatments every 6 h. The samples of culture medium obtained were stored at -20°C before being assayed for PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  by radioimmunoassay (section 2:5). After culture, the pieces of tissue were removed from each Petri dish into preweighed containers and dried by placing in an oven at 37°C for 24 h. Each container was then reweighed and the amount of dry tissue from each Petri dish was calculated. The outputs of prostaglandins were calculated per mg dry weight of tissue per 6 h.

### Statistical Tests

Changes in the output of PGs with time were analysed by Duncan’s multiple range test. Differences between treated and control groups were analyzed by Student’s t-test or, if the variances of the two groups were significantly different by the variance ratio F test, by a modified t-test for unequal variances.

## Results and Discussion

### A. Basal PG outputs during culture

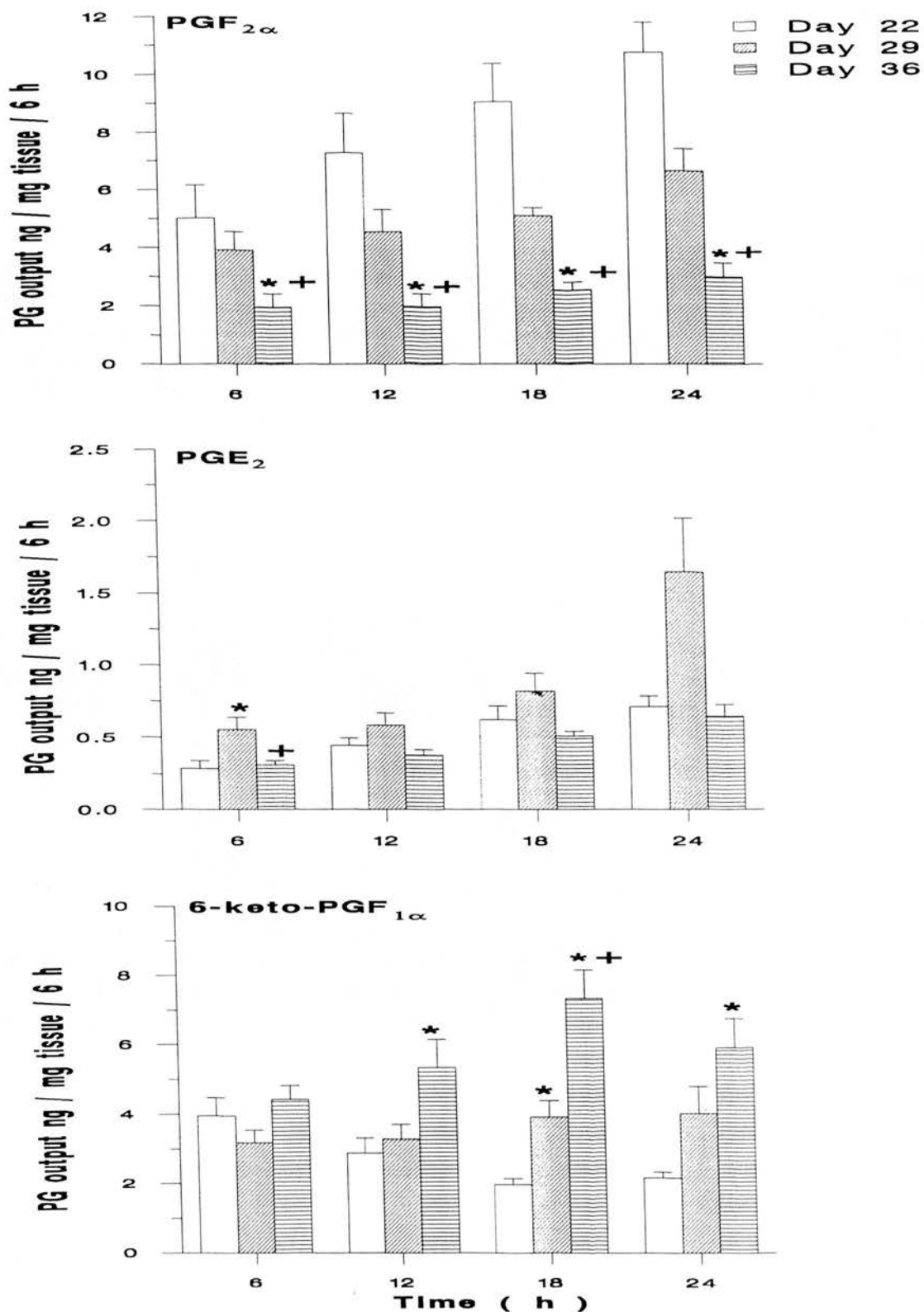
#### Results

##### Basal PG outputs from the endometrium

The basal outputs of endometrial  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 22, 29 and 36 pregnant guinea-pigs during 24 h culture are illustrated in Figure 38. Whilst endometrial  $\text{PGF}_{2\alpha}$  output was seen to decrease during pregnancy (i.e.  $\text{PGF}_{2\alpha}$  output on day 22 > day 29 > day 36 pregnant animals), 6-keto- $\text{PGF}_{1\alpha}$  output increased during pregnancy (i.e. 6-keto- $\text{PGF}_{1\alpha}$  output on day 36 > day 29 > day 22 pregnant animals). In comparison to measurements on day 22 of pregnancy,  $\text{PGF}_{2\alpha}$  output was significantly ( $P < 0.05$ ) lower on day 29 of pregnancy at 18 h and on day 36 of pregnancy at 6, 12, 18 and 24 h of culture. Endometrial  $\text{PGF}_{2\alpha}$  output was also significantly ( $P < 0.05$ ) lower on day 36 compared to day 29 of pregnancy at 6, 12, 18 and 24 h of culture.  $\text{PGE}_2$  output was not significantly different at 12, 18 and 24 h of culture on any day of pregnancy, although output from day 29 endometrium appeared to be highest. Indeed, at 6 h of culture  $\text{PGE}_2$  output was significantly ( $P < 0.05$ ) greater from day 29 compared to day 22 pregnant endometrium, and significantly ( $P < 0.05$ ) lower from day 36 compared to day 29 pregnant endometrium. Endometrial 6-keto- $\text{PGF}_{1\alpha}$  output was significantly ( $P < 0.05$ ) greater on day 29 of pregnancy at 18 h and on day 36 of pregnancy at 12, 18 and 24 h of culture compared to 6-keto- $\text{PGF}_{1\alpha}$  output on day 22 of pregnancy. At 18 h of culture, 6-keto- $\text{PGF}_{1\alpha}$  output was also significantly ( $P < 0.05$ ) greater in day 36, compared to day 29, pregnant guinea-pigs.

Endometrial  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs tended to increase during culture on all days of pregnancy. The output of 6-keto- $\text{PGF}_{1\alpha}$  from day 22 pregnant animals appeared to decrease during culture, whilst output tended to increase during culture in day 29 and day 36 pregnant animals.





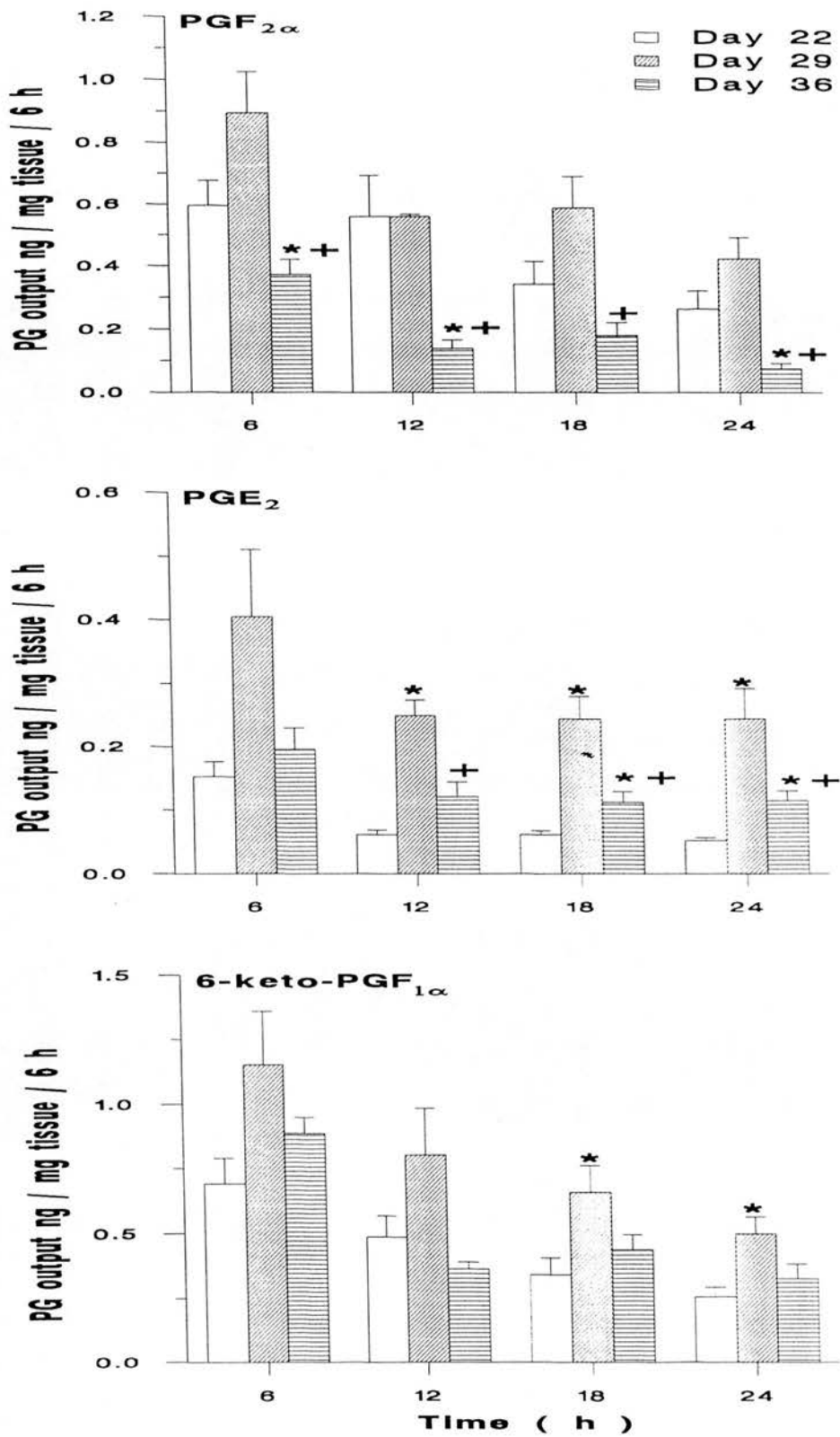
**Figure 38.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from endometrium of day 22, 29 and 36 pregnant guinea-pigs cultured for 24 h and sampled every 6 h. Significantly ( $P < 0.05$ ) different to day 22 (\*) or day 29 (+) value for the same PG at the same time.

#### Basal PG outputs from the subplacenta

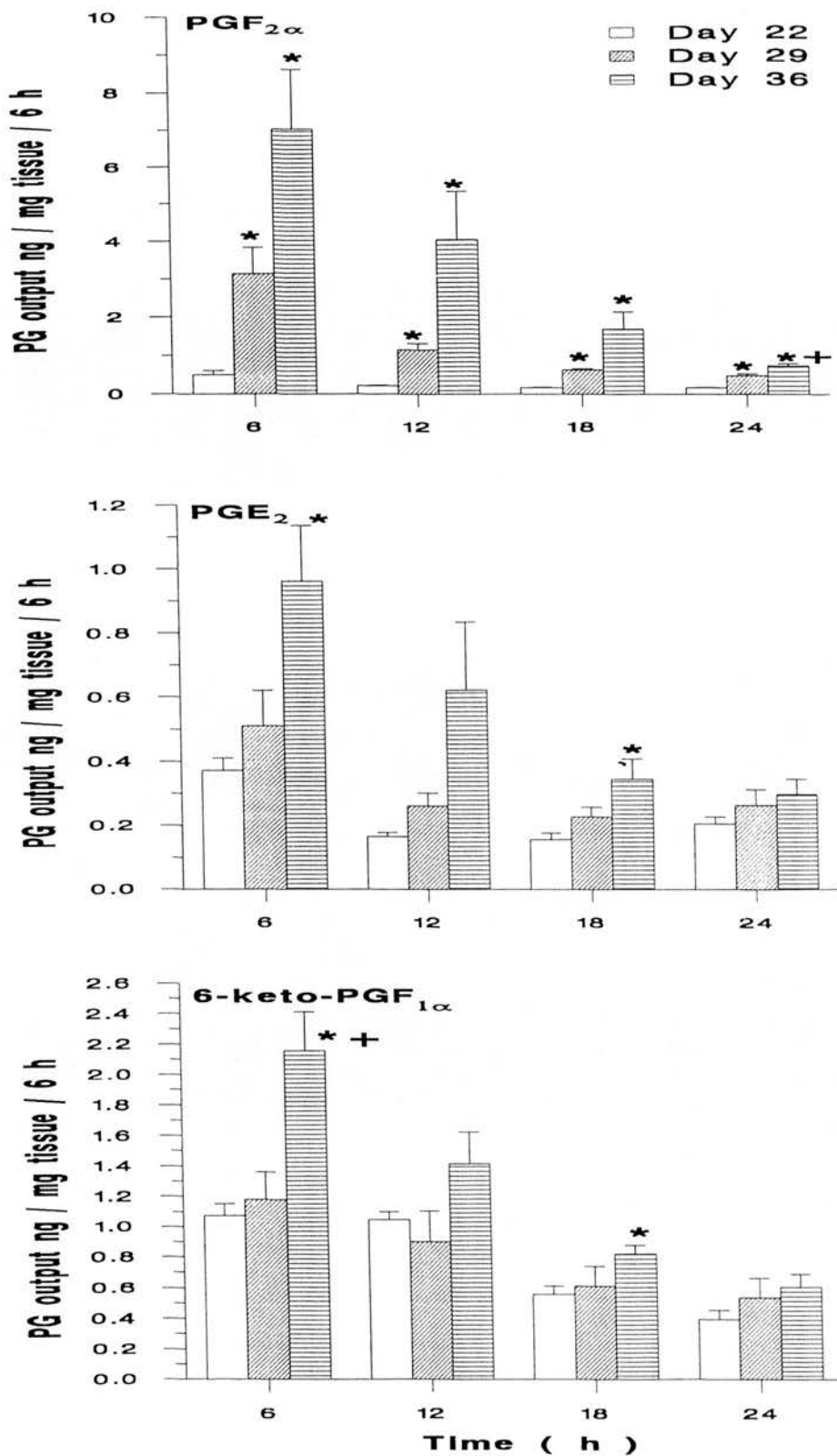
The basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the subplacenta of day 22, 29 and 36 pregnant guinea-pigs during 24 h tissue culture are illustrated in Figure 39.  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs from the subplacenta all appeared to be greatest on day 29 of pregnancy.  $\text{PGF}_{2\alpha}$  output was significantly ( $P < 0.05$ ) lower in day 36, compared to day 22 pregnant animals, at 6, 12 and 24 h of culture and in day 36, compared to day 29 pregnant animals, at 6, 12, 18 and 24 h of culture.  $\text{PGE}_2$  output was significantly greater from day 29 pregnant animals at 12, 18 and 24 h, and from day 36 pregnant animals at 18 and 24 h of culture, compared to  $\text{PGE}_2$  output from day 22 pregnant guinea-pigs. However,  $\text{PGE}_2$  output was significantly lower on day 36, compared to day 29 of pregnancy, at 12, 18 and 24 h of culture. The output of 6-keto- $\text{PGF}_{1\alpha}$  was similar on all days of pregnancy, except for a significant increase in output on day 29, compared to day 22 of pregnancy, at 18 and 24 h of culture. The outputs of all the prostaglandins tended to decrease during culture.

#### Basal PG outputs from the chorio-allantoic placenta

The basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the chorio-allantoic placenta of day 22, 29 and 36 pregnant guinea-pigs during 24 h tissue culture are illustrated in Figure 40. All PG outputs from the chorio-allantoic placenta in culture increased from day 22 to day 36 of pregnancy.  $\text{PGF}_{2\alpha}$  output was significantly ( $P < 0.05$ ) greater on day 29 of pregnancy at 6, 12, 18 and 24 h and on day 36 of pregnancy at 6, 12, 18 and 24 h of culture, compared to  $\text{PGF}_{2\alpha}$  output on day 22 of pregnancy.  $\text{PGF}_{2\alpha}$  output was also significantly ( $P < 0.05$ ) greater on day 36, compared to day 29, of pregnancy at 24 h of culture.  $\text{PGE}_2$  output increased throughout pregnancy, but these increases were only significant on day 36, compared to day 22, of pregnancy at 6 and 18 h ( $P < 0.05$ ) of culture. Similarly, 6-keto- $\text{PGF}_{1\alpha}$  output tended to increase throughout pregnancy, but these increases were only significant ( $P < 0.05$ ) on day 36, compared to day 22, of pregnancy at 6 and 24 h of culture and on day 36, compared to day 29, of pregnancy at 6 h of culture. All PG outputs on all days of pregnancy tended to decrease during culture.



**Figure 39.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the subplacenta of day 22, 29 and 36 pregnant guinea-pigs cultured for 24 h and sampled every 6 h. Significantly ( $P < 0.05$ ) different to day 22 (\*) or day 29 (+) value for the same PG at the same time.



**Figure 40.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the chorio-allantoic placenta of day 22, 29 and 36 pregnant guinea-pigs cultured for 24 h and sampled every 6 h. Significantly ( $P < 0.05$ ) different to day 22 (\*) or day 29 (+) value for the same PG at the same time.

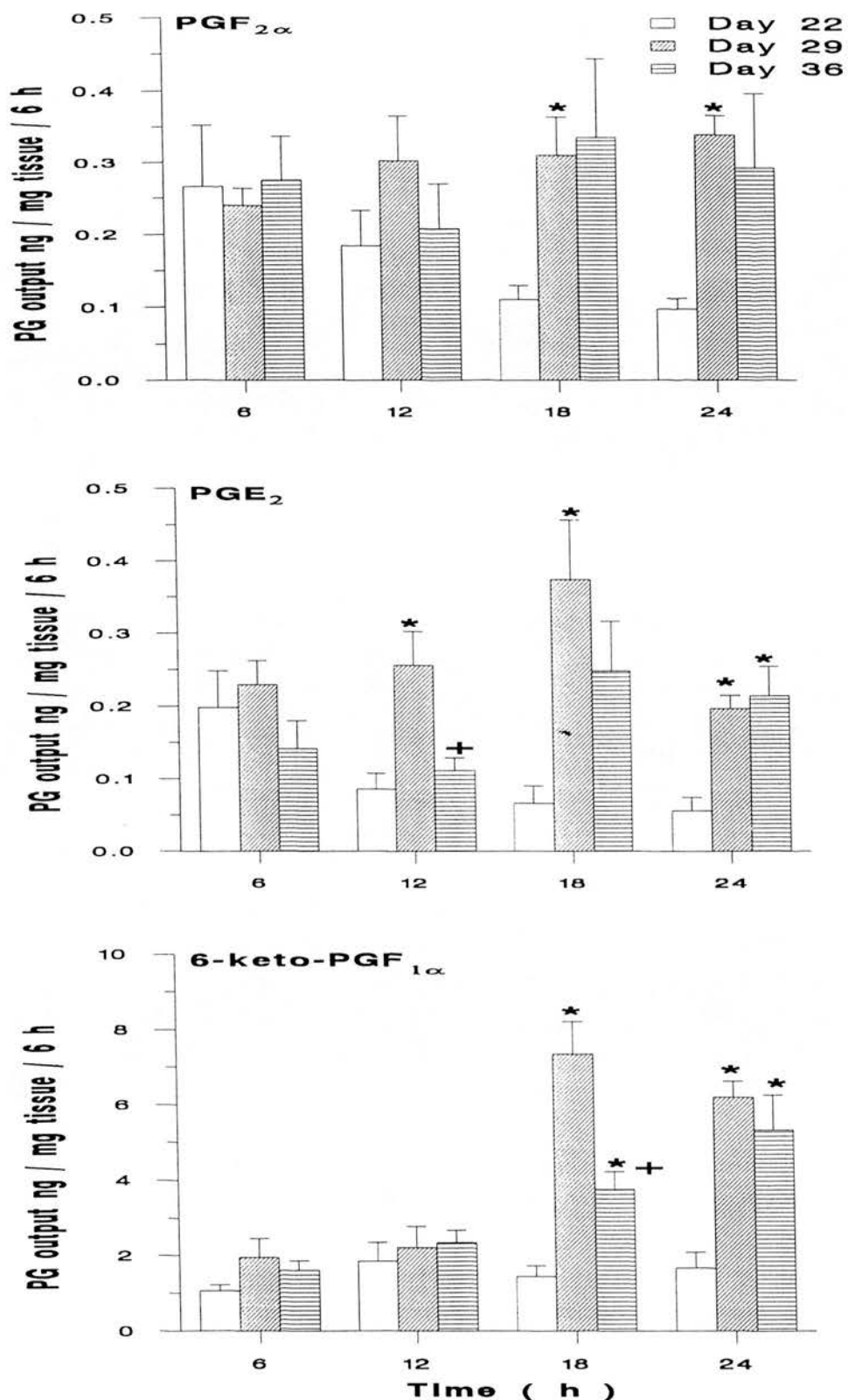
### Basal PG outputs from the fetal membranes

The basal outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the fetal membranes of day 22, 29 and 36 pregnant guinea-pigs during 24 h tissue culture are illustrated in Figure 41. PG outputs tended to be higher in late pregnant guinea-pigs; this was especially true at 18 and 24 h of culture.  $\text{PGF}_{2\alpha}$  output was significantly ( $P < 0.05$ ) greater on day 29, compared to day 22, of pregnancy at 18 and 24 h of culture.  $\text{PGE}_2$  output was significantly ( $P < 0.05$ ) greater on day 29 of pregnancy at 12, 18 and 24 h and on day 36 of pregnancy at 18 and 24 h of culture compared to  $\text{PGE}_2$  output on day 22 of pregnancy.  $\text{PGE}_2$  output was significantly ( $P < 0.05$ ) lower on day 36 compared to day 29 of pregnancy at 12 h of culture. The output of 6-keto- $\text{PGF}_{1\alpha}$  was significantly ( $P < 0.05$ ) greater in day 29 pregnant animals at 18 and 24 h of culture and in day 36 pregnant animals at 18 and 24 h of culture, compared to day 22 pregnant guinea-pigs. At 18 h of culture, 6-keto- $\text{PGF}_{1\alpha}$  output was significantly ( $P < 0.05$ ) lower in day 36, compared to day 29, pregnant animals. All PG outputs on day 22 of pregnancy tended to decrease during culture, whilst on day 29 and 36 of pregnancy, PG outputs tended to increase during culture.

### Discussion

In previous tissue culture experiments it has been demonstrated that the presence of indomethacin in the culture medium greatly inhibits the output of all three PGs (Riley and Poyser, 1990), indicating that PG synthesis and secretion from guinea-pig endometrium occurs during tissue culture. In the above experiment all basal PG outputs from the endometrium, particularly  $\text{PGF}_{2\alpha}$ , increased during 24 h tissue culture. This may result from the removal of the inhibitory influence of progesterone on endometrial  $\text{PGF}_{2\alpha}$  synthesis in the guinea-pig (Leaver and Seawright, 1982; Riley and Poyser, 1987a), due to the removal of the tissue from the animal. It is also clear that endometrial  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs decreased and increased, respectively during pregnancy (i.e. between days 22 and 36 of pregnancy) in the guinea-pig. Therefore, a certain amount of redistribution from  $\text{PGF}_{2\alpha}$  synthesis to 6-keto- $\text{PGF}_{1\alpha}$  synthesis may occur as gestation advances.

The outputs of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  were generally greatest from the endometrium (when compared at 6 h of culture). However, there was a striking 14-



**Figure 41.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the fetal membranes of day 22, 29 and 36 pregnant guinea-pigs cultured for 24 h and sampled every 6 h. Significantly ( $P < 0.05$ ) different to day 22 (\*) or day 29 (+) value for the same PG at the same time.



fold increase in  $\text{PGF}_{2\alpha}$  output between days 22 and 36 of pregnancy in the chorio-allantoic placenta (when compared at 6 h of culture).  $\text{PGE}_2$  output was similar in all tissues. The results indicate that in the pregnant guinea-pig, the endometrium continues to be a major source of prostaglandins, with the chorio-allantoic placenta having a role in increased prostaglandin, particularly  $\text{PGF}_{2\alpha}$ , output later in pregnancy.

## **B. Effects of THM and ARA treatment on endometrial PG outputs**

### **Results**

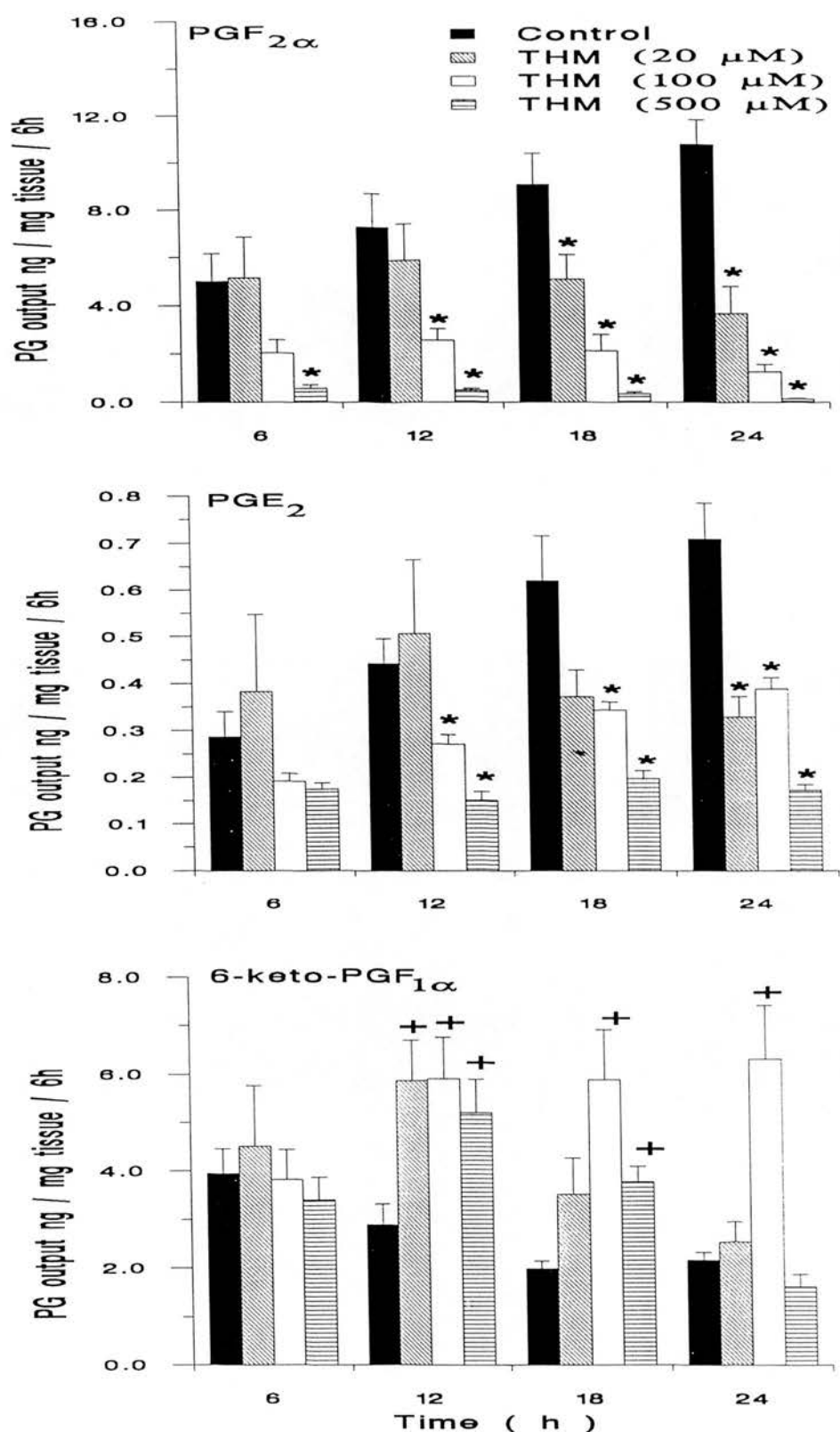
#### **i. Endometrium from day 22 pregnant animals**

The effects of THM and ARA treatment on endometrial PG outputs from day 22 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 42 and 43.

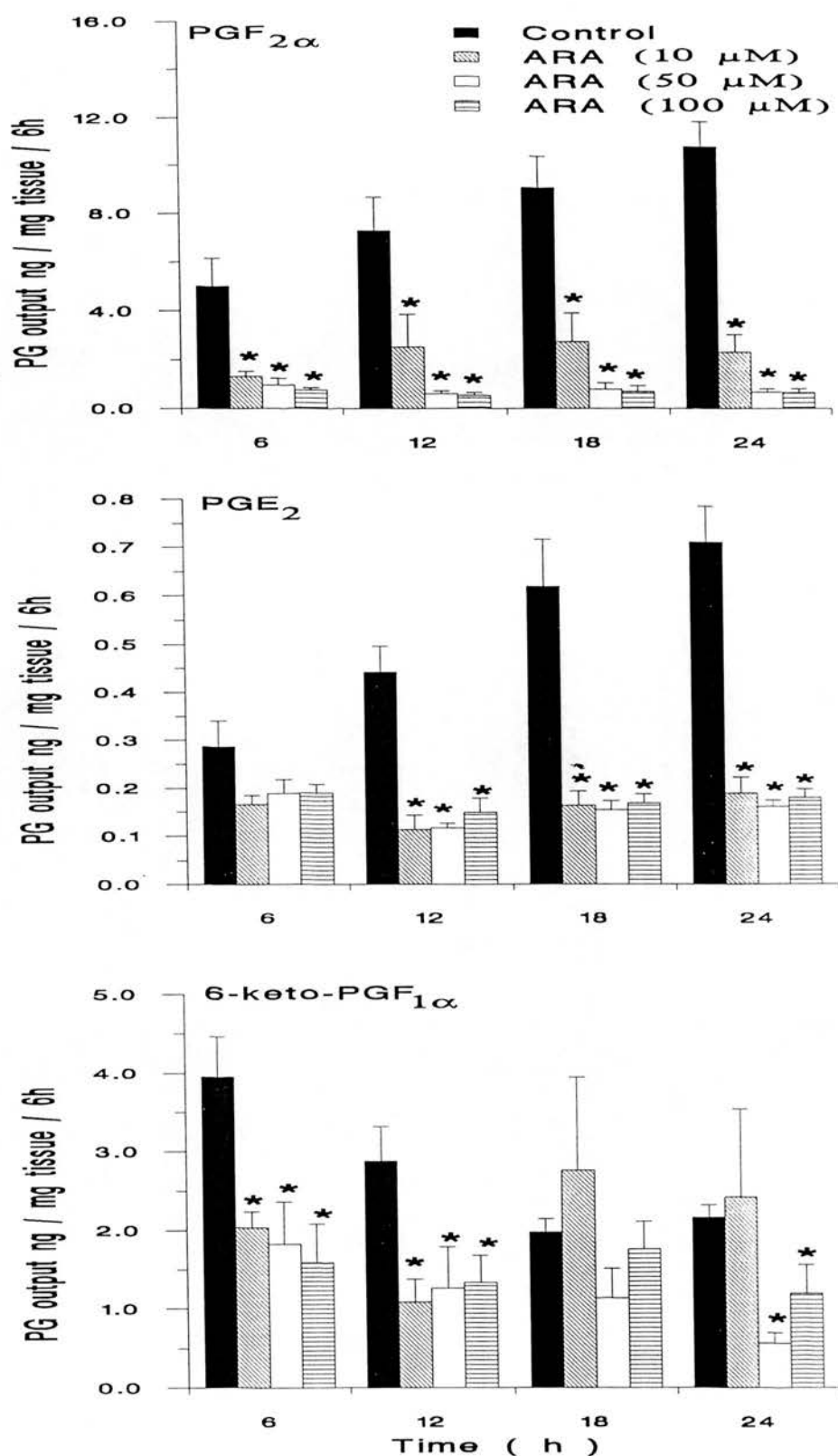
*PGF<sub>2α</sub> output* -  $\text{PGF}_{2\alpha}$  output was significantly ( $P < 0.05$ ) decreased by 500  $\mu\text{M}$  THM at 6, 12, 18 and 24 h, by 100  $\mu\text{M}$  THM at 12, 18 and 24 h, and by 20  $\mu\text{M}$  THM at 18 and 24 h of culture. 10, 50 and 100  $\mu\text{M}$  ARA all significantly ( $P < 0.05$ ) decreased  $\text{PGF}_{2\alpha}$  output at 6 h of culture.  $\text{PGF}_{2\alpha}$  output was also significantly ( $P < 0.05$ ) decreased by 100, 50 and 10  $\mu\text{M}$  ARA at 12, 18 and 24 h of culture.

*PGE<sub>2</sub> output* - Both THM and ARA treatment inhibited  $\text{PGE}_2$  output; these decreases were significant at 12, 18 and 24 h of culture. 500 and 100  $\mu\text{M}$  THM significantly ( $P < 0.05$ ) decreased  $\text{PGE}_2$  output at 12, 18 and 24 h of culture, and 20  $\mu\text{M}$  THM significantly ( $P < 0.05$ ) decreased  $\text{PGE}_2$  output at 24 h of culture. 100, 50 and 10  $\mu\text{M}$  ARA significantly ( $P < 0.05$ ) decreased  $\text{PGE}_2$  output at 12, 18 and 24 h of culture.

*6-keto-PGF<sub>1α</sub> output* - 6-keto- $\text{PGF}_{1\alpha}$  output was significantly ( $P < 0.05$ ) increased by THM treatment at 12 h by 500, 100 and 20  $\mu\text{M}$ , at 18 h by 500 and 100  $\mu\text{M}$  and at 24 h of culture by 100  $\mu\text{M}$  THM. Treatment of endometrium with ARA decreased 6-keto- $\text{PGF}_{1\alpha}$  output; these decreases were significant at 6, 12 and 24 h of culture. 6-keto- $\text{PGF}_{1\alpha}$  output was significantly ( $P < 0.05$ ) decreased by 100  $\mu\text{M}$  ARA at 6, 12 and 24 h, by 50  $\mu\text{M}$  ARA at 6, 12 and 24 h, and by 10  $\mu\text{M}$  ARA at 6 and 12 h of culture.



**Figure 42.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 22 pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500  $\mu\text{M}$ ). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.



**Figure 43.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 22 pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM). \* Significantly ( $P < 0.05$ ) lower than the control value for the same PG at the same time.

## ii. Endometrium from day 29 pregnant animals

The effects of THM and ARA treatment on endometrial PG outputs from day 29 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 44 and 45.

*PGF<sub>2α</sub> output* - PGF<sub>2α</sub> output was significantly ( $P < 0.05$ ) decreased by 500  $\mu\text{M}$  THM at 6, 12, 18 and 24 h, by 100  $\mu\text{M}$  THM at 6, 12, 18 and 24 h, and by 20  $\mu\text{M}$  THM at 6, 18 and 24 h of culture. All concentrations of ARA at all times of culture significantly ( $P < 0.05$ ) decreased PGF<sub>2α</sub> output.

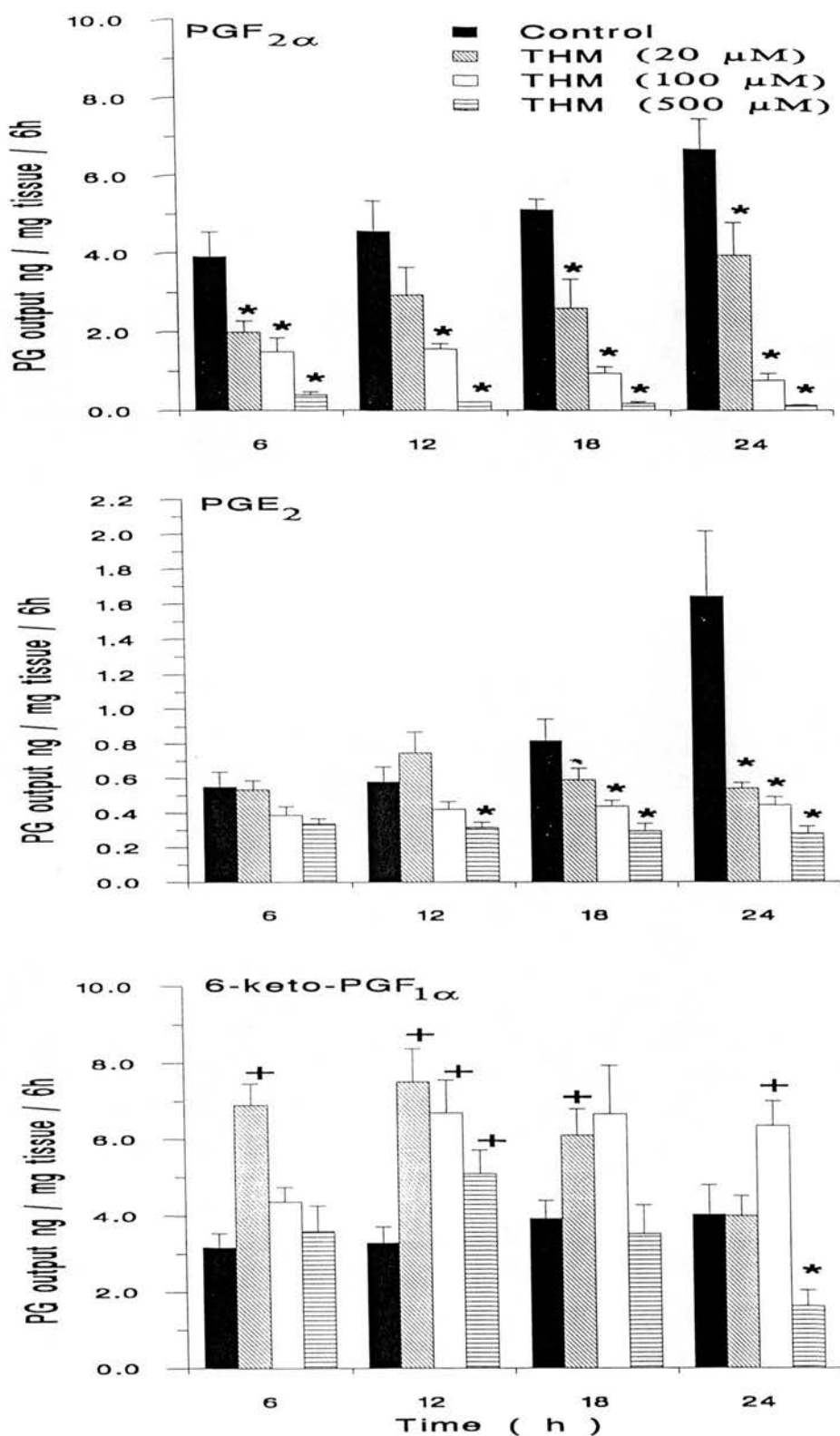
*PGE<sub>2</sub> output* - THM treatment decreased PGE<sub>2</sub> output in general. Significant ( $P < 0.05$ ) decreases resulted from treatment with 500  $\mu\text{M}$  THM at 12, 18 and 24 h, with 100  $\mu\text{M}$  THM at 18 and 24 h, and with 20  $\mu\text{M}$  THM at only 24 h of culture. PGE<sub>2</sub> output was also significantly ( $P < 0.05$ ) decreased by 100  $\mu\text{M}$  and 50  $\mu\text{M}$  ARA at 18 and 24 h, and by 10  $\mu\text{M}$  ARA at 12 and 24 h of culture.

*6-keto-PGF<sub>1α</sub> output* - 20  $\mu\text{M}$  THM significantly ( $P < 0.05$ ) increased 6-keto-PGF<sub>1α</sub> output at 6, 12 and 18 h of culture. 100  $\mu\text{M}$  THM also significantly ( $P < 0.05$ ) increased output at 12 and 24 h of culture. 500  $\mu\text{M}$  THM significantly ( $P < 0.05$ ) increased and decreased 6-keto-PGF<sub>1α</sub> output at 12 and 24 h of culture, respectively. ARA treatment tended to decrease endometrial 6-keto-PGF<sub>1α</sub> output from day 29 pregnant animals. These decreases were significant ( $P < 0.05$ ) following treatment with 10, 50 and 100  $\mu\text{M}$  at 12 h, and with 50  $\mu\text{M}$  and 100  $\mu\text{M}$  at 18 h and 24 h of culture.

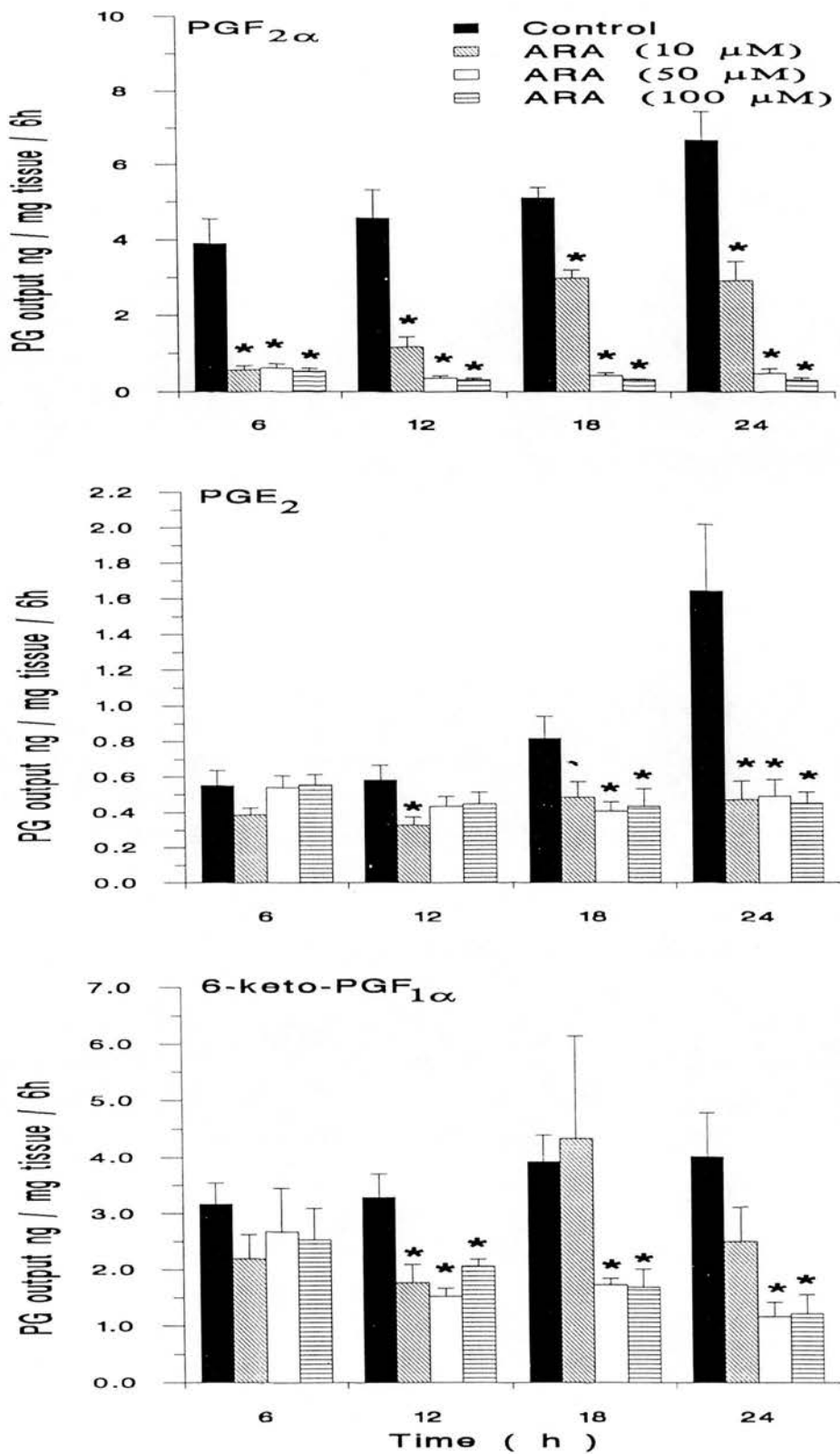
## iii. Endometrium from day 36 pregnant animals

The effects of THM and ARA treatment on endometrial PG outputs from day 36 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 46 and 47.

*PGF<sub>2α</sub> output* - Both THM and ARA treatment decreased endometrial PGF<sub>2α</sub> output on day 36 of pregnancy. These decreases were significant ( $P < 0.05$ ) with 500  $\mu\text{M}$  and 100  $\mu\text{M}$  ARA at 6, 12, 18 and 24 h, with 100  $\mu\text{M}$  THM at 18 and 24 h, with 20  $\mu\text{M}$  THM at 18 h, with 50  $\mu\text{M}$  ARA at 6 and 24 h, and with 10  $\mu\text{M}$  ARA at 18 and 24 h of culture.

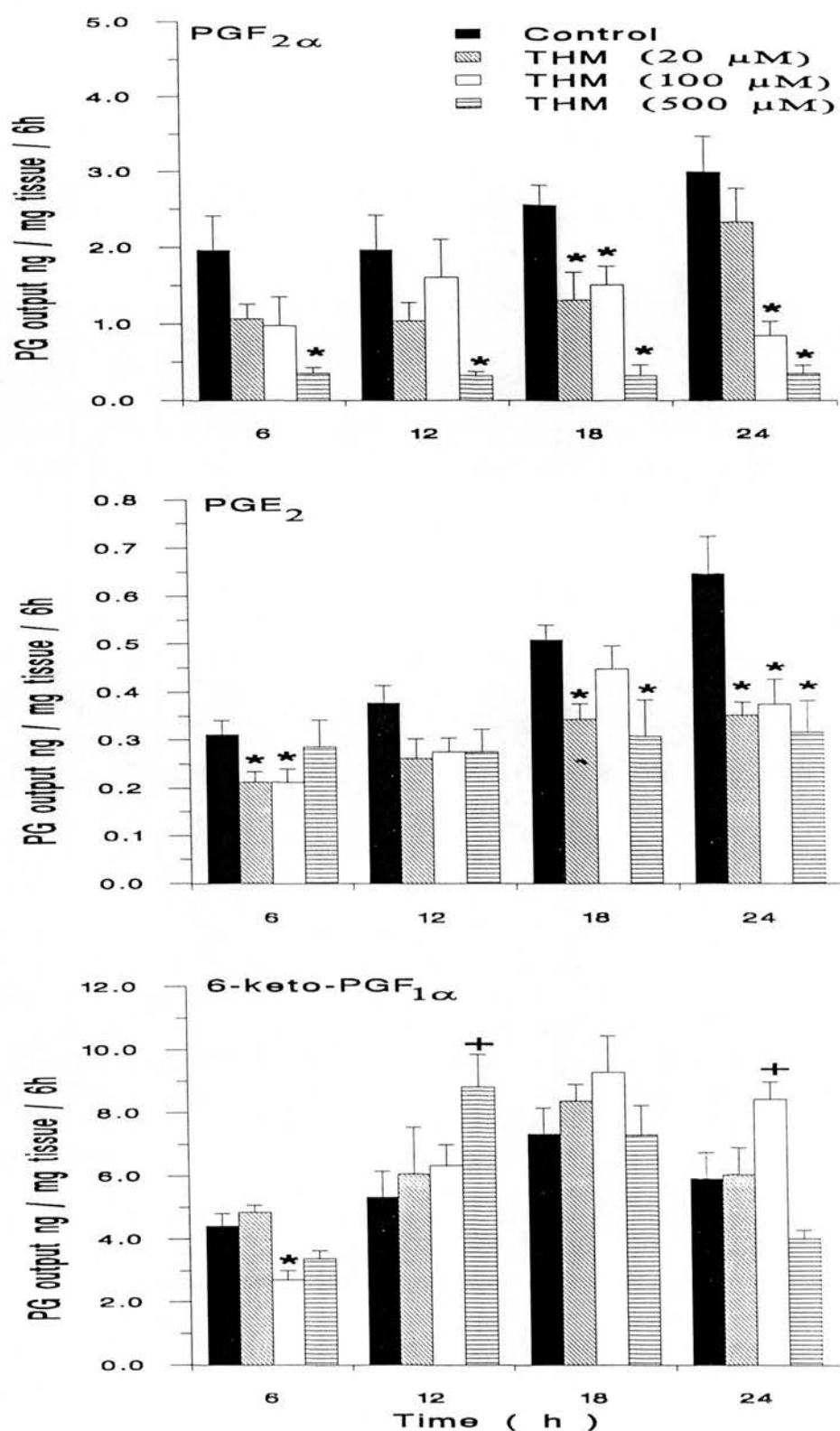


**Figure 44.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 29 pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500  $\mu\text{M}$ ). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.

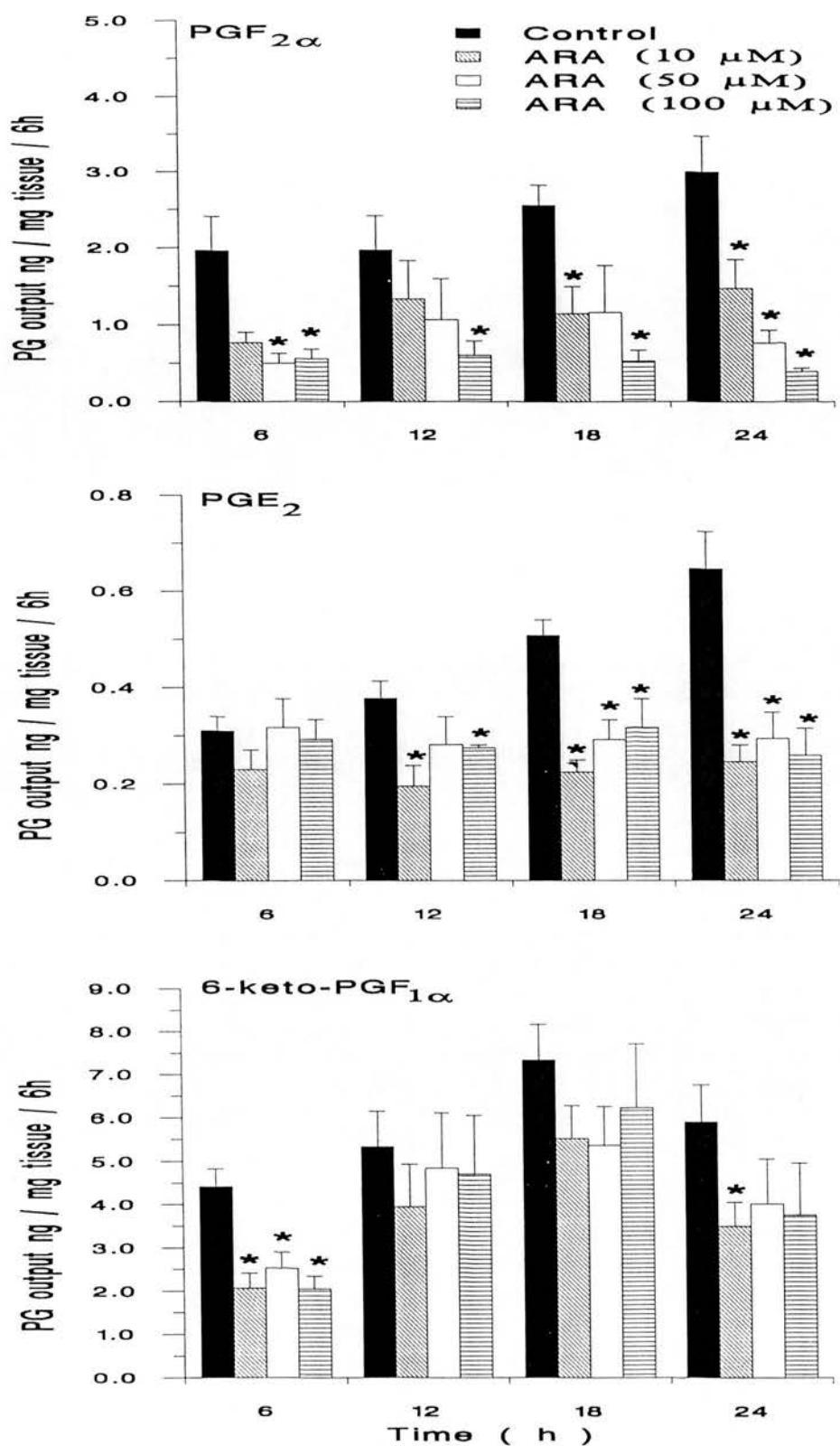


**Figure 45.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 29 pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM). \* Significantly ( $P < 0.05$ ) lower than the control value for the same PG at the same time.





**Figure 46.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from day 36 pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500  $\mu\text{M}$ ). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.



**Figure 47.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from day 36 pregnant guinea-pig endometrium cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM). \* Significantly ( $P < 0.05$ ) lower than the control value for the same PG at the same time.

*PGE<sub>2</sub> output* - Both THM and ARA treatment tended to decrease endometrial PGE<sub>2</sub> output on day 36 of pregnancy. PGE<sub>2</sub> output was significantly ( $P < 0.05$ ) decreased by 20  $\mu$ M THM at 6, 18 and 24 h, by 100  $\mu$ M THM at 6 and 24 h, by 500  $\mu$ M THM at 18 and 24 h, by 10  $\mu$ M ARA at 12, 18 and 24 h, by 50  $\mu$ M ARA at 18 and 24 h, and by 100  $\mu$ M ARA at 12, 18 and 24 h of culture.

*6-keto-PGF<sub>1 $\alpha$</sub>  output* - Variable changes in 6-keto-PGF<sub>1 $\alpha$</sub>  output due to THM treatment were measured during culture, since 100  $\mu$ M THM significantly ( $P < 0.05$ ) decreased output at 6 h, but 500  $\mu$ M and 100  $\mu$ M THM significantly ( $P < 0.05$ ) increased output at 12 and 24 h of culture, respectively. Similarly to, but not as dramatically as PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> outputs, 6-keto-PGF<sub>1 $\alpha$</sub>  output was decreased by ARA treatment. At 6 h of culture, 6-keto-PGF<sub>1 $\alpha$</sub>  output was significantly ( $P < 0.05$ ) decreased by 10, 50 and 100  $\mu$ M ARA and at 24 h of culture by 10  $\mu$ M ARA.

## **Discussion**

On all days of pregnancy, treatment with THM (an inhibitor of arachidonic acid reacylation) resulted in a reduction in the outputs of both endometrial PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub>. In contrast, 6-keto-PGF<sub>1 $\alpha$</sub>  output was increased by THM treatment. ARA, the PLA<sub>2</sub> inhibitor, decreased the outputs of all PGs on all days of pregnancy illustrating the essential role that PLA<sub>2</sub> plays in the control of the release of free arachidonic acid for prostaglandin production by the guinea-pig endometrium.

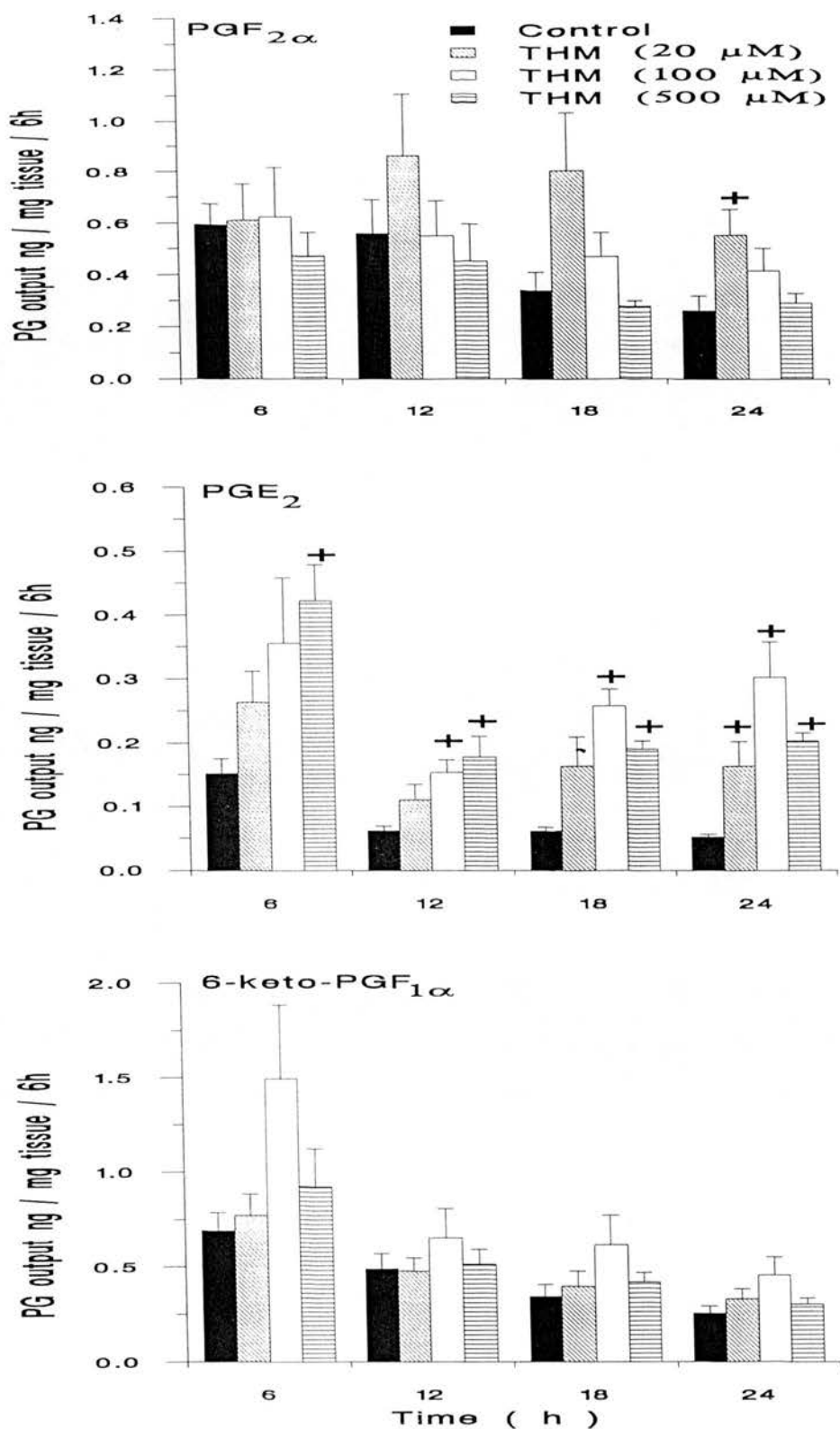
## **C. Effects of THM and ARA treatment on PG output from the subplacenta**

### **Results**

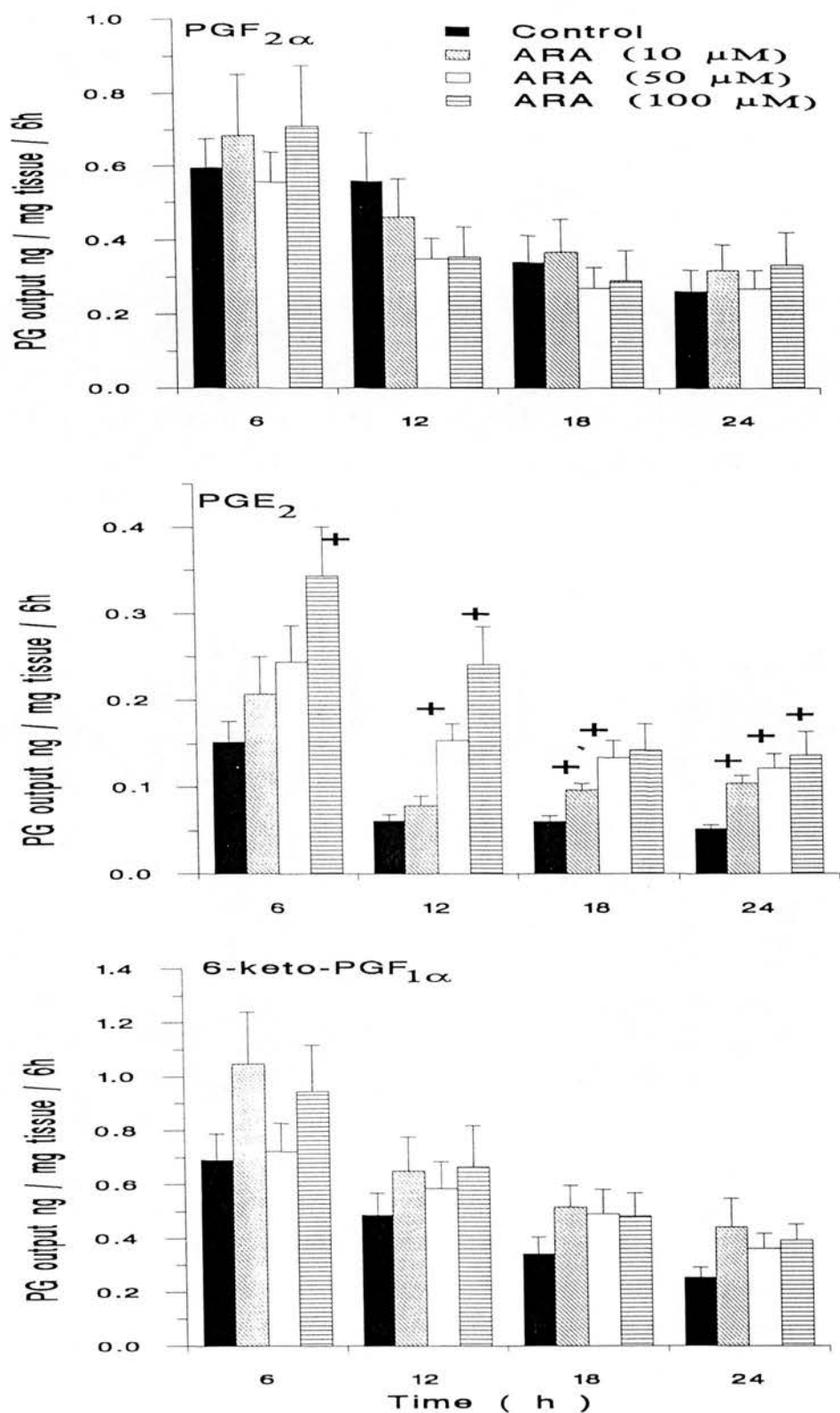
#### **i. Subplacenta from day 22 pregnant animals**

The effects of THM and ARA treatment on PG outputs from the subplacenta of day 22 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 48 and 49.

*PGF<sub>2 $\alpha$</sub>  output* - PGF<sub>2 $\alpha$</sub>  output from the subplacenta of early (day 22) pregnant guinea-pigs appeared to be generally unaffected by either THM or ARA treatment. However, a slight potentiation in output did appear to occur following treatment with



**Figure 48.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the subplacenta of day 22 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500 μM).  
 + Significantly ( $P < 0.05$ ) higher than the control value for the same PG at the same time.



**Figure 49.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the subplacenta of day 22 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM).  
 + Significantly ( $P < 0.05$ ) higher than the control value for the same PG at the same time.

THM (20  $\mu$ M, but not 100 and 500  $\mu$ M) at 12, 18 and 24 h of culture; this potentiation was significant ( $P < 0.05$ ) at 24 h of culture.

*PGE<sub>2</sub> output* - PGE<sub>2</sub> output was significantly ( $P < 0.05$ ) increased with 20  $\mu$ M THM at 24 h, with 100  $\mu$ M THM at 12, 18 and 24 h, and with 500  $\mu$ M THM at 6, 12, 18 and 24 h of culture. ARA treatment dose-dependently increased PGE<sub>2</sub> output; significant ( $P < 0.05$ ) increases were measured with 10  $\mu$ M ARA at 18 and 24 h, with 50  $\mu$ M ARA at 12, 18 and 24 h, and with 100  $\mu$ M ARA at 6, 12 and 24 h of culture.

*6-keto-PGF<sub>1 $\alpha$</sub>  output* - The output of 6-keto-PGF<sub>1 $\alpha$</sub>  did not significantly change following either THM or ARA treatment. However, both inhibitors did appear to slightly potentiate 6-keto-PGF<sub>1 $\alpha$</sub>  output; this was particularly true of 100  $\mu$ M THM at all times during culture.

#### ii. Subplacenta from day 29 pregnant animals

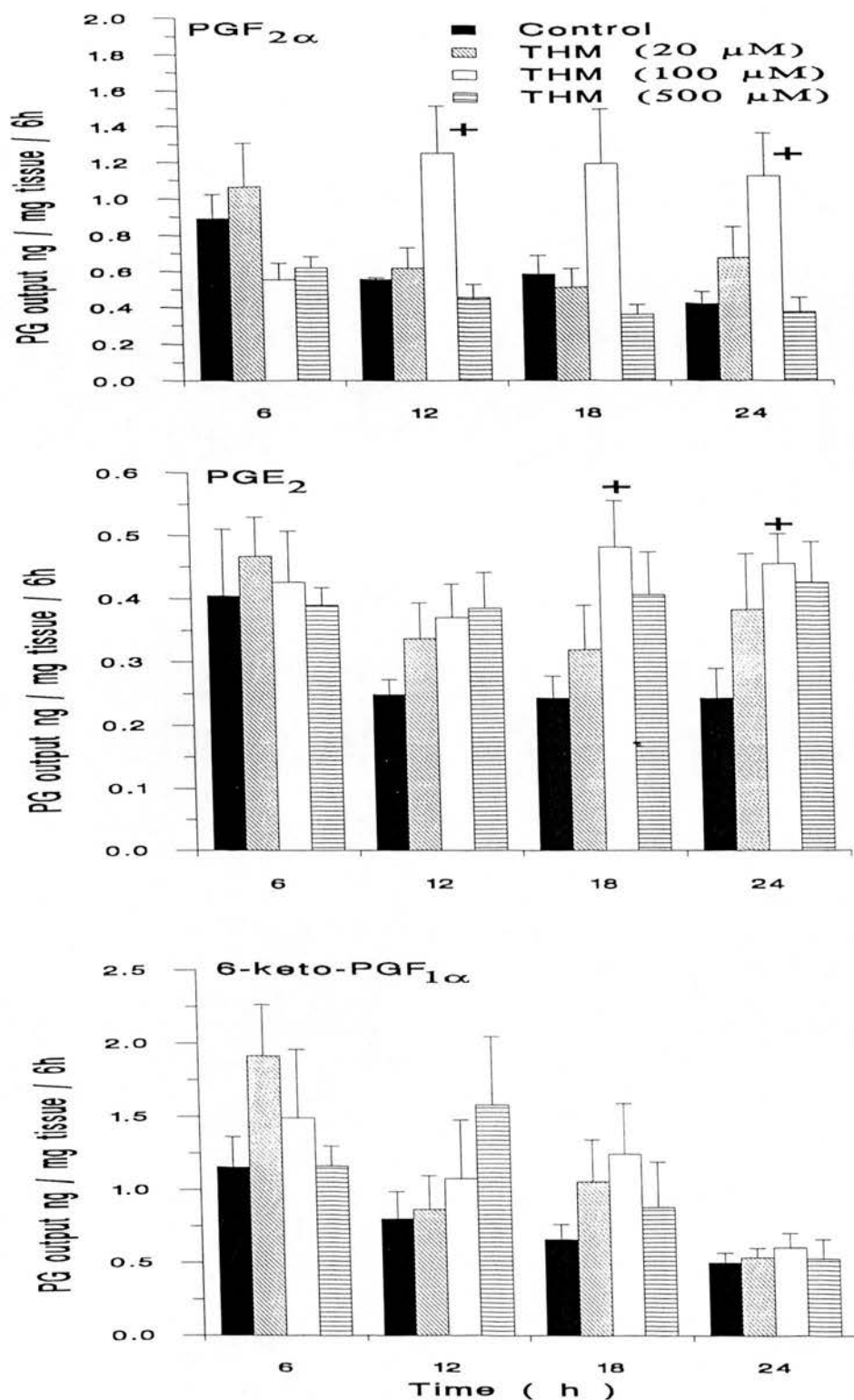
The effects of THM and ARA treatment on PG outputs from the subplacenta of day 29 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 50 and 51.

*PGF<sub>2 $\alpha$</sub>  output* - PGF<sub>2 $\alpha$</sub>  output was generally unaffected by either THM or ARA treatment, except for 100  $\mu$ M THM which increased PGF<sub>2 $\alpha$</sub>  output at 12, 18 and 24 h of culture, although the increase was significant ( $P < 0.05$ ) only at 12 and 24 h. Also, PGF<sub>2 $\alpha$</sub>  output was significantly decreased by 50 and 100  $\mu$ M ARA at 12 h of culture ( $P < 0.05$ ).

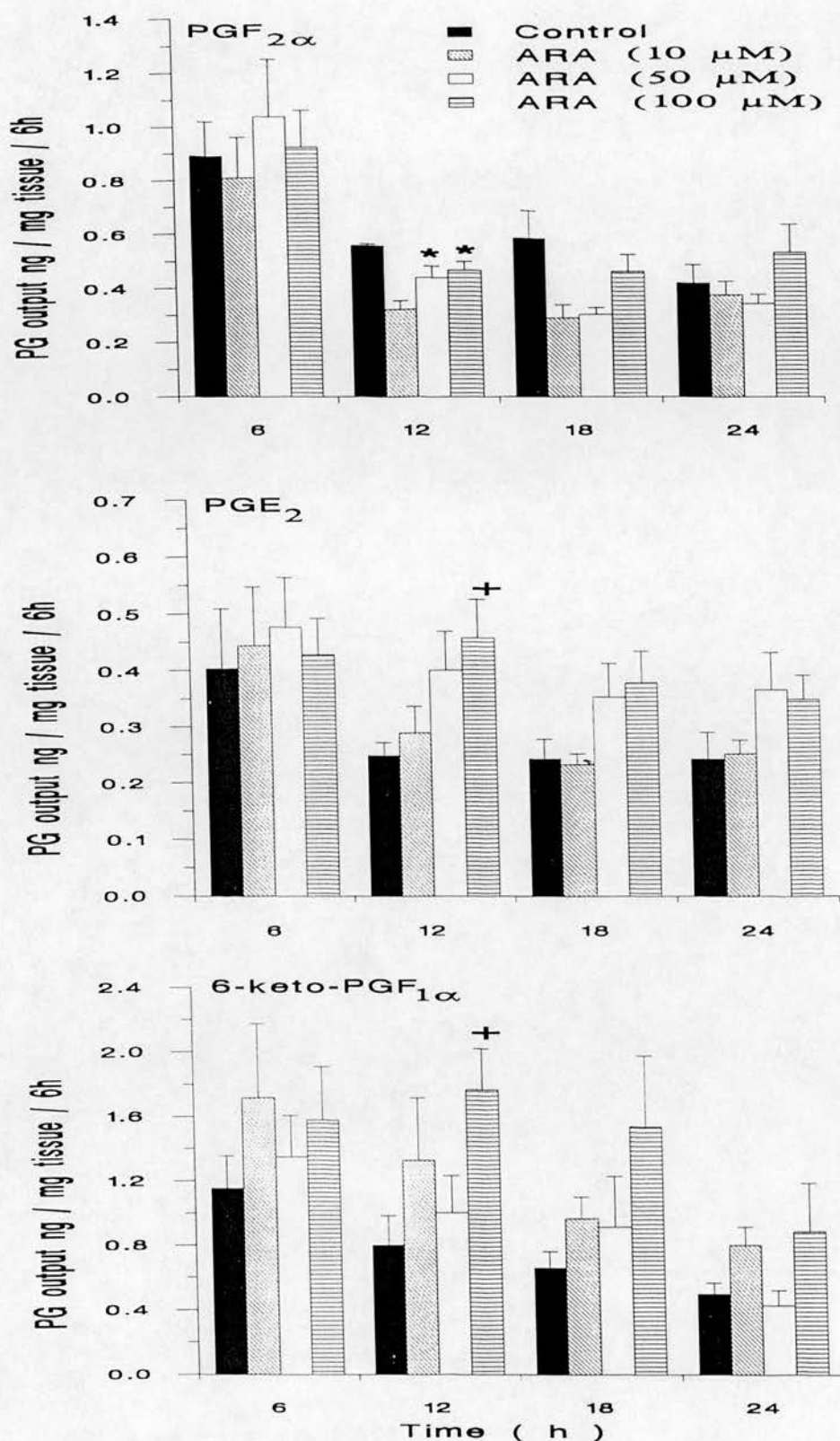
*PGE<sub>2</sub> output* - PGE<sub>2</sub> output was generally increased following THM or ARA treatment. Increases were significant ( $P < 0.05$ ) with 100  $\mu$ M THM at 18 and 24 h, and with 100  $\mu$ M ARA at 12 h of culture.

*6-keto-PGF<sub>1 $\alpha$</sub>  output* - No significant changes in 6-keto-PGF<sub>1 $\alpha$</sub>  output were measured following either THM or ARA treatment of subplacenta tissue from day 29 pregnant guinea-pigs, except for 100  $\mu$ M ARA which significantly increased 6-keto-PGF<sub>1 $\alpha$</sub>  output ( $P < 0.05$ ) at 12 h of culture.





**Figure 50.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the subplacenta of day 29 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500 μM).  
<sup>+</sup> Significantly ( $P < 0.05$ ) higher than the control value for the same PG at the same time.



**Figure 51.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the subplacenta of day 29 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.

### iii. Subplacenta from day 36 pregnant animals

The effects of THM and ARA treatment on PG outputs from the subplacenta of day 36 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 52 and 53.

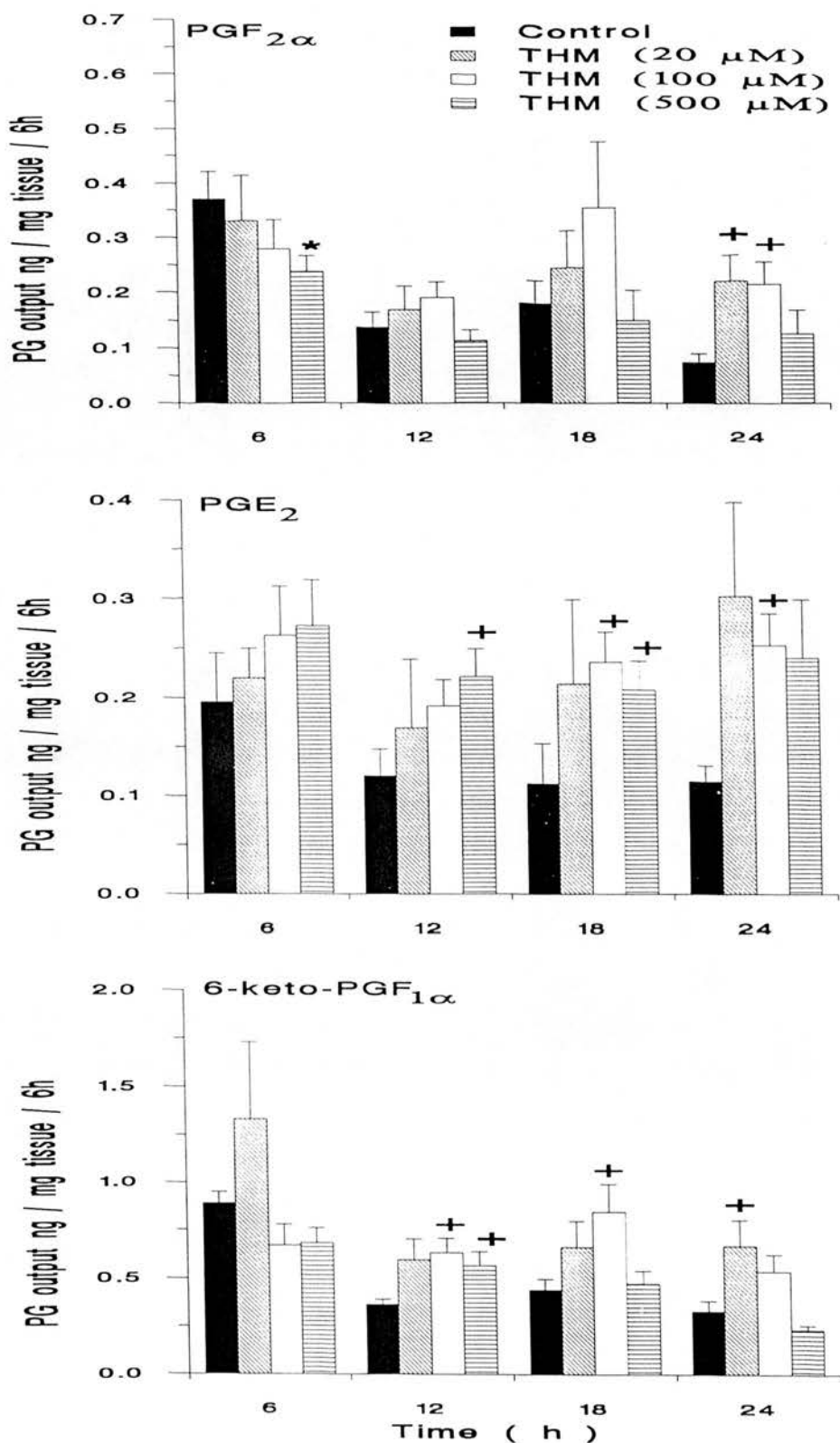
*PGF<sub>2α</sub> output* - The output of PGF<sub>2α</sub> appeared generally unaffected following either THM or ARA treatment, except with 500 μM THM which significantly ( $P < 0.05$ ) decreased PGF<sub>2α</sub> output at 6 h, and with 20 and 100 μM THM which significantly ( $P < 0.05$ ) increased PGF<sub>2α</sub> output at 24 h of culture.

*PGE<sub>2</sub> output* - Both THM and ARA treatment appeared to increase PGE<sub>2</sub> output. However, this was only to a significant ( $P < 0.05$ ) level with 100 μM THM at 18 and 24 h, and with 500 μM THM at 12 and 18 h of culture.

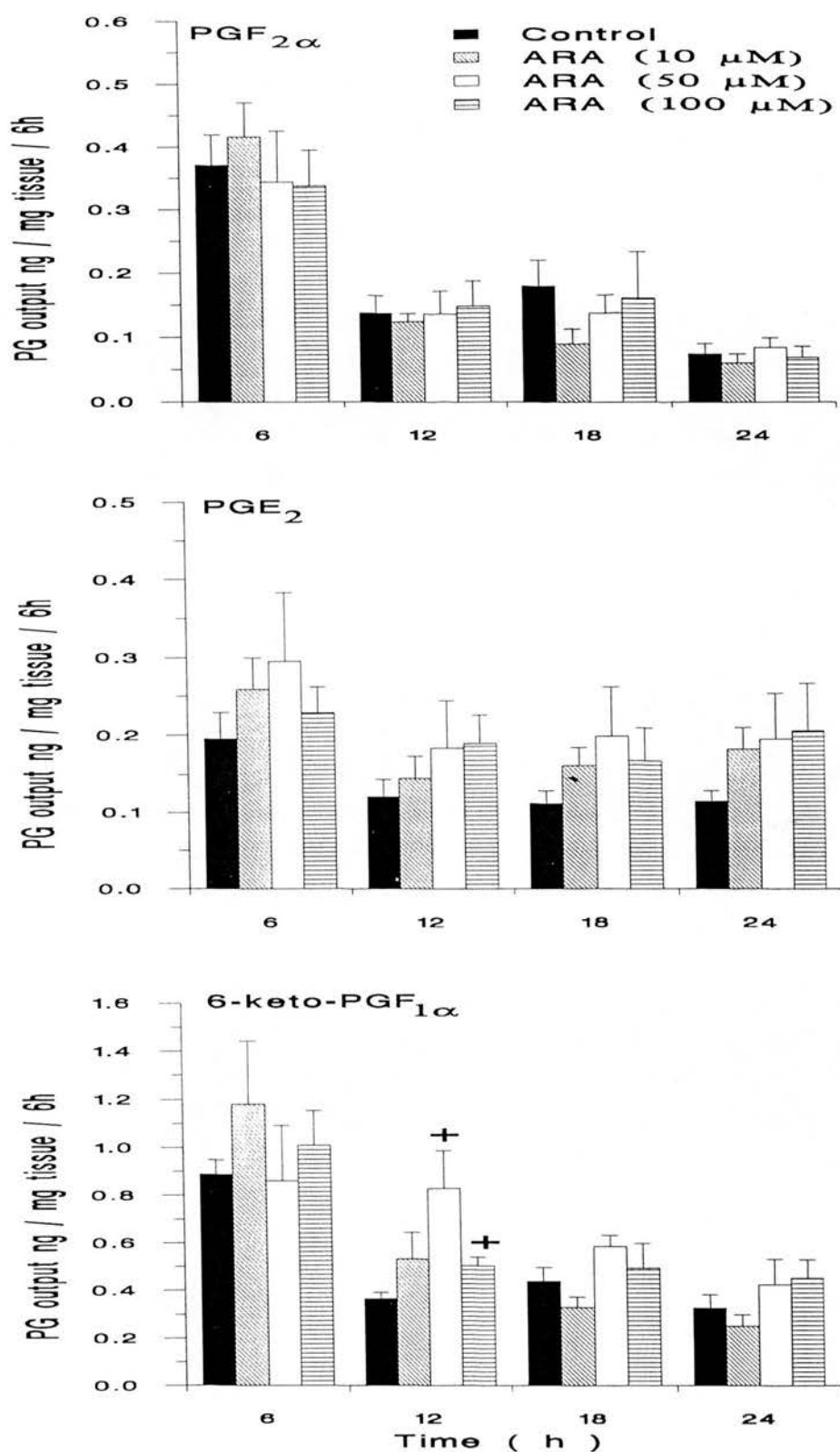
*6-keto-PGF<sub>1α</sub> output* - Significant ( $P < 0.05$ ) increases in 6-keto-PGF<sub>1α</sub> output were measured with 20 μM THM at 24 h, with 100 μM THM at 12 and 18 h, with 500 μM THM at 12 h, and with 50 μM and 100 μM ARA at 12 h of culture.

### **Discussion**

In contrast to the results demonstrated in the endometrium, PGF<sub>2α</sub> and PGE<sub>2</sub> outputs tended to be increased slightly by an inhibitor (THM) of the reacylation of arachidonic acid. The output of 6-keto-PGF<sub>1α</sub> was generally unaffected by THM treatment, except for a slight increase on day 36 of pregnancy. ARA treatment had no effect on PGF<sub>2α</sub> output (except for a slight decrease in day 29 pregnant animals) indicating that PLA<sub>2</sub> is not involved in the control of PGF<sub>2α</sub> synthesis from the subplacenta. Unexpected results were found as with regard to PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs; ARA treatment increased PGE<sub>2</sub> output on days 22 and 29 of pregnancy and increased 6-keto-PGF<sub>1α</sub> on days 29 and 36 of pregnancy.



**Figure 52.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the subplacenta of day 36 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500 μM). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.



**Figure 53.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the subplacenta of day 36 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM). + Significantly ( $P < 0.05$ ) higher than the control value for the same PG at the same time.

## **D. Effects of THM and ARA treatment on PG output from the chorio-allantoic placenta**

### **Results**

#### **i. Chorio-allantoic placenta from day 22 pregnant animals**

The effects of THM and ARA treatment on PG outputs from the chorio-allantoic placenta of day 22 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 54 and 55.

*PGF<sub>2α</sub> output* - PGF<sub>2α</sub> output was unaffected by THM or ARA treatment, except for a significant decrease with 10 μM ARA at 12 h of culture ( $P < 0.05$ ).

*PGE<sub>2</sub> output* - PGE<sub>2</sub> output was generally potentiated by THM treatment at 12, 18 and 24 h of culture. Increases were significant ( $P < 0.05$ ) with 20, 100 and 500 μM THM at 18 and 24 h of culture. ARA treatment tended to decrease PGE<sub>2</sub> output at low concentrations and increase PGE<sub>2</sub> output at high concentrations. However, the only significant ( $P < 0.05$ ) change measured was the decrease in output at 6 and 12 h of culture.

*6-keto-PGF<sub>1α</sub> output* - 6-keto-PGF<sub>1α</sub> output was significantly ( $P < 0.05$ ) increased with 100 μM THM at 18 and 24 h, and with 50 μM ARA at 6 h of culture.

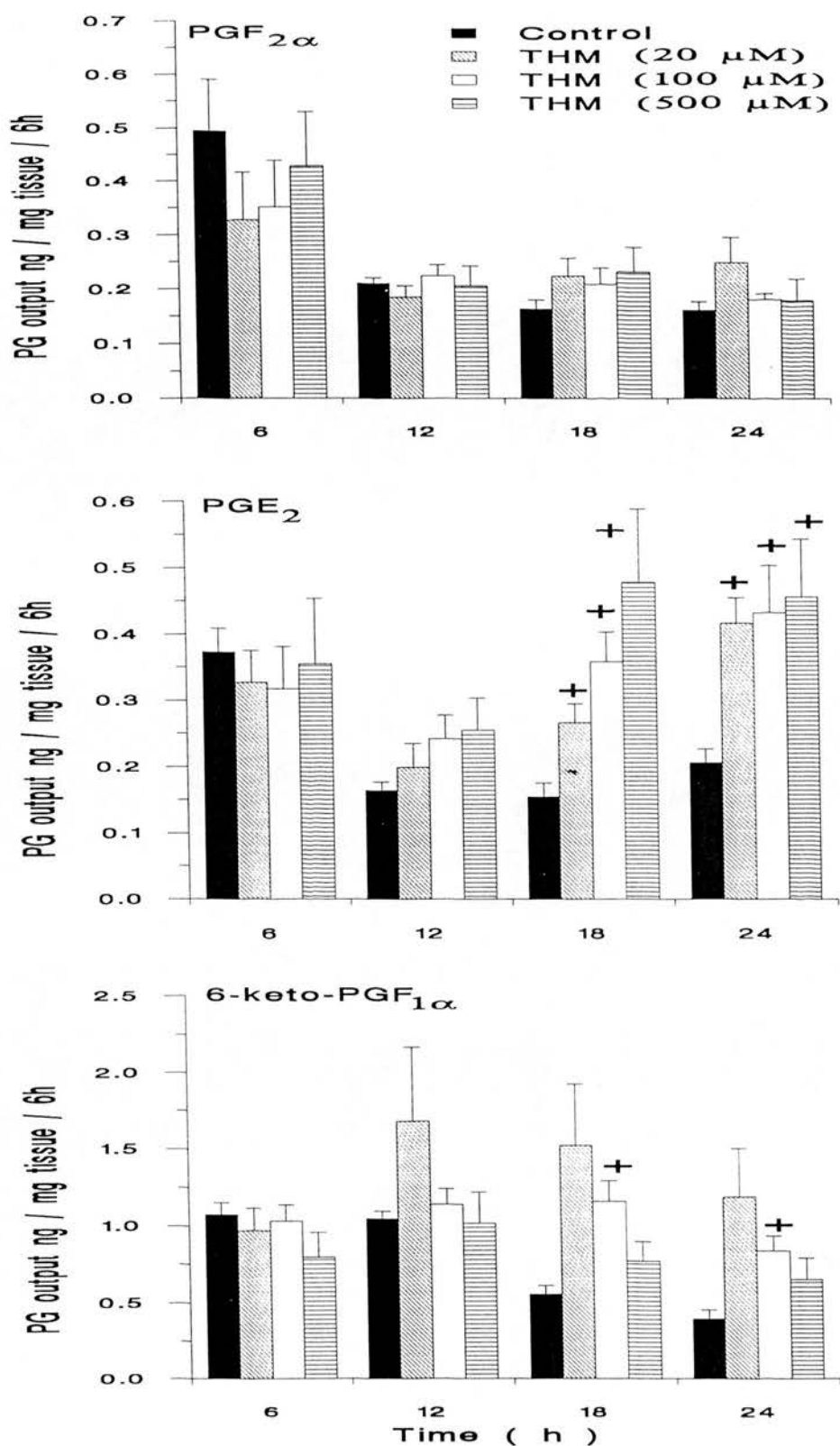
#### **ii. Chorio-allantoic placenta from day 29 pregnant animals**

The effects of THM and ARA treatment on PG outputs from the chorio-allantoic placenta of day 29 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 56 and 57.

*PGF<sub>2α</sub> output* - THM treatment potentiated PGF<sub>2α</sub> output at 12, 18 and 24 h of culture. Increases were significant ( $P < 0.05$ ) at 24 h with 100 and 500 μM THM. PGF<sub>2α</sub> output was unaffected by ARA treatment.

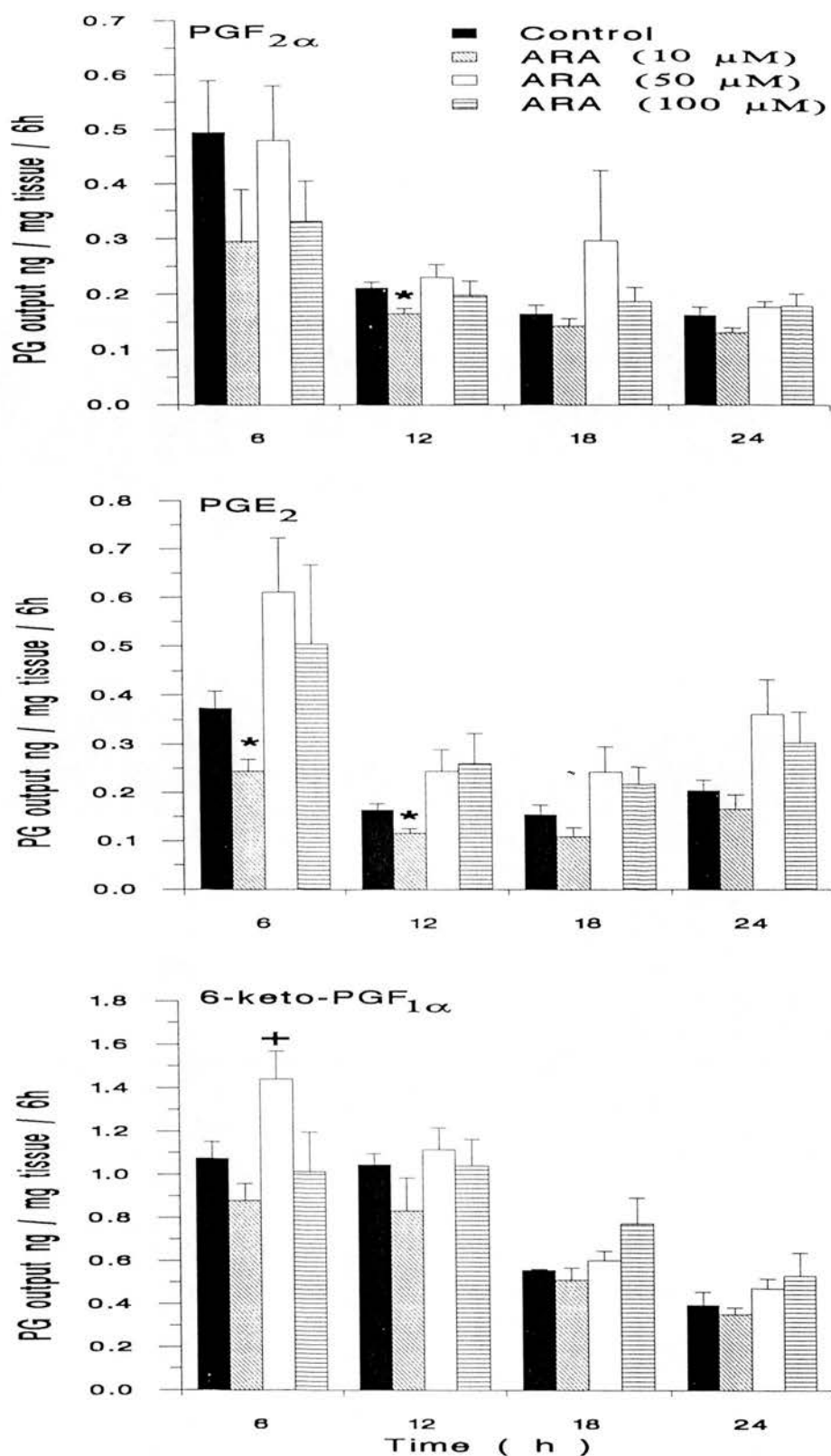
*PGE<sub>2</sub> output* - THM treatment potentiated PGE<sub>2</sub> output at 12, 18 and 24 h of culture. Increases were significant ( $P < 0.05$ ) with 100 μM THM at 18 and 24 h of culture. PGE<sub>2</sub> output was unaffected by ARA treatment.

*6-keto-PGF<sub>1α</sub> output* - The output of 6-keto-PGF<sub>1α</sub> was generally unaffected by both THM and ARA treatment, except with 500 μM THM at 24 h and with 10 μM ARA at 12 h of culture which both significantly ( $P < 0.05$ ) increased 6-keto-PGF<sub>1α</sub> output.

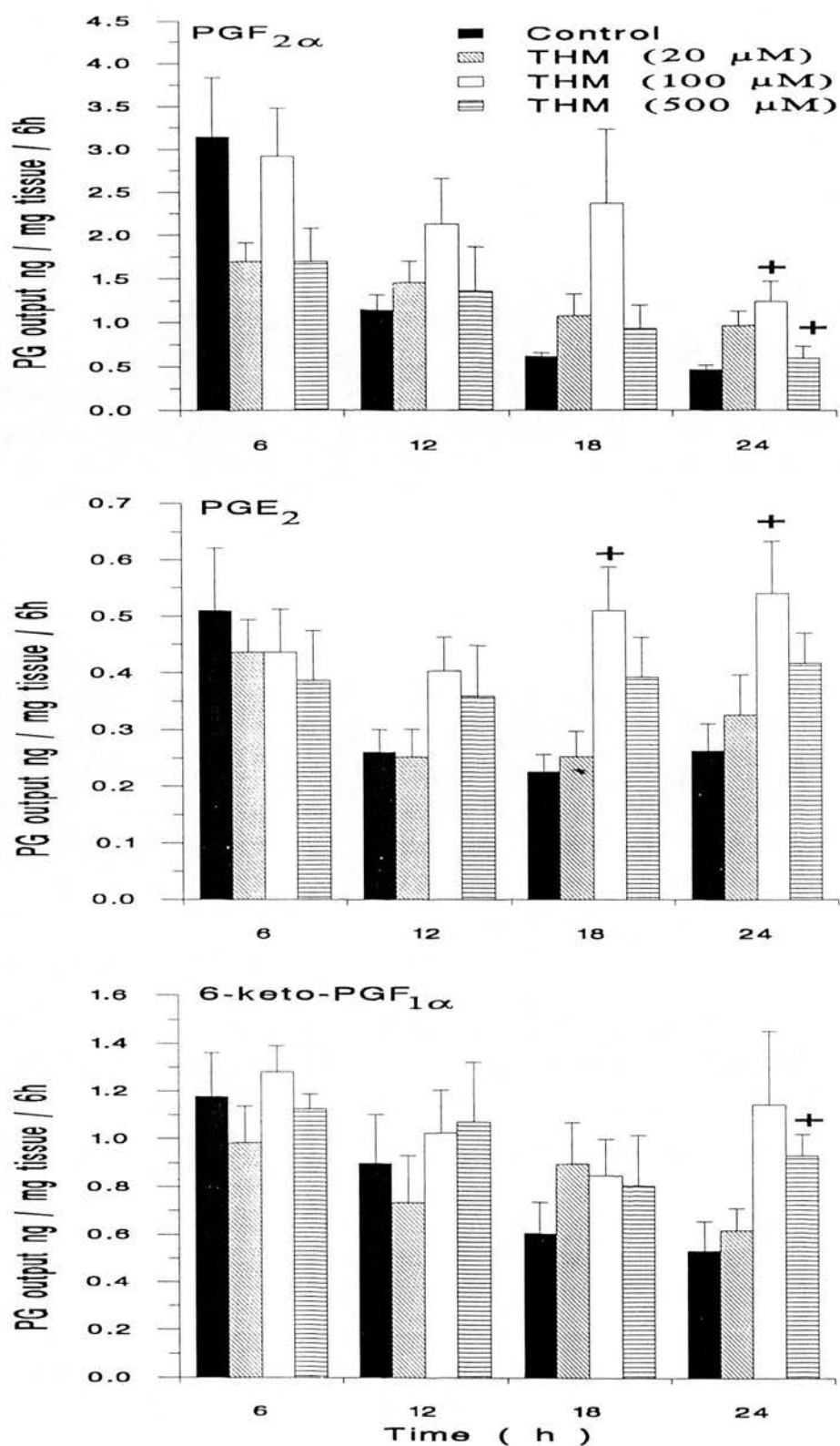


**Figure 54.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the chorio-allantoic placenta of day 22 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500 μM). + Significantly ( $P < 0.05$ ) higher than the control value for the same PG at the same time.

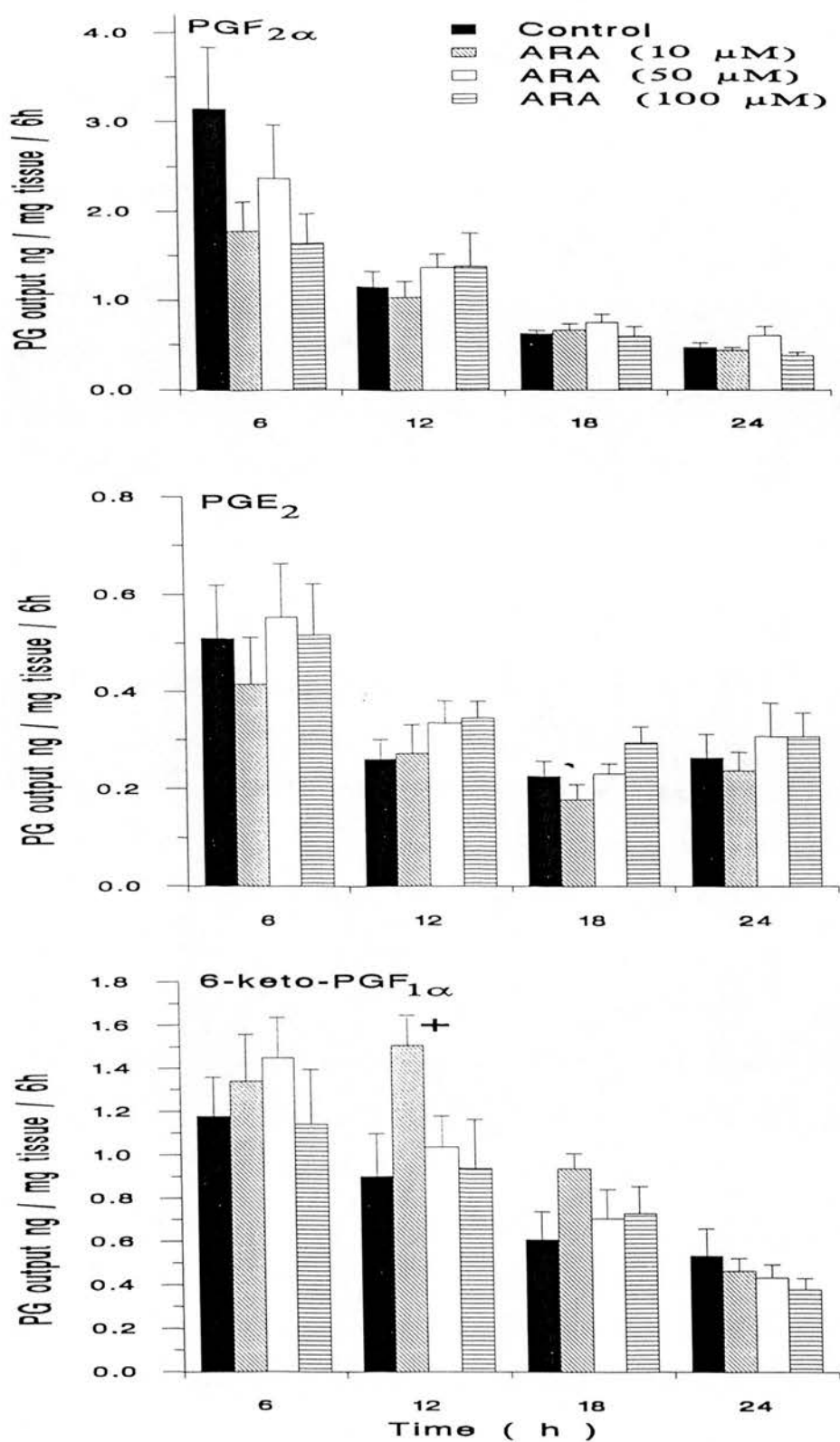




**Figure 55.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the chorio-allantoic placenta of day 22 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.



**Figure 56.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the chorio-allantoic placenta of day 29 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500 μM). + Significantly ( $P < 0.05$ ) higher than the control value for the same PG at the same time.



**Figure 57.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the chorio-allantoic placenta of day 29 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM). + Significantly ( $P < 0.05$ ) higher than the control value for the same PG at the same time.

### iii. Chorio-allantoic placenta from day 36 pregnant animals

The effects of THM and ARA treatment on PG outputs from the chorio-allantoic placenta of day 36 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 58 and 59.

*PGF<sub>2α</sub> output* - THM treatment resulted in little change in PGF<sub>2α</sub> output at 6, 12 and 18 h, but significant ( $P < 0.05$ ) increases in PGF<sub>2α</sub> output occurred at 24 h of culture following 100 and 500  $\mu\text{M}$  THM treatment. ARA treatment tended to potentiate PGF<sub>2α</sub> output, and significant ( $P < 0.05$ ) increases occurred following 10  $\mu\text{M}$  ARA treatment at 18 and 24 h, and 50  $\mu\text{M}$  ARA treatment at 24 h of culture.

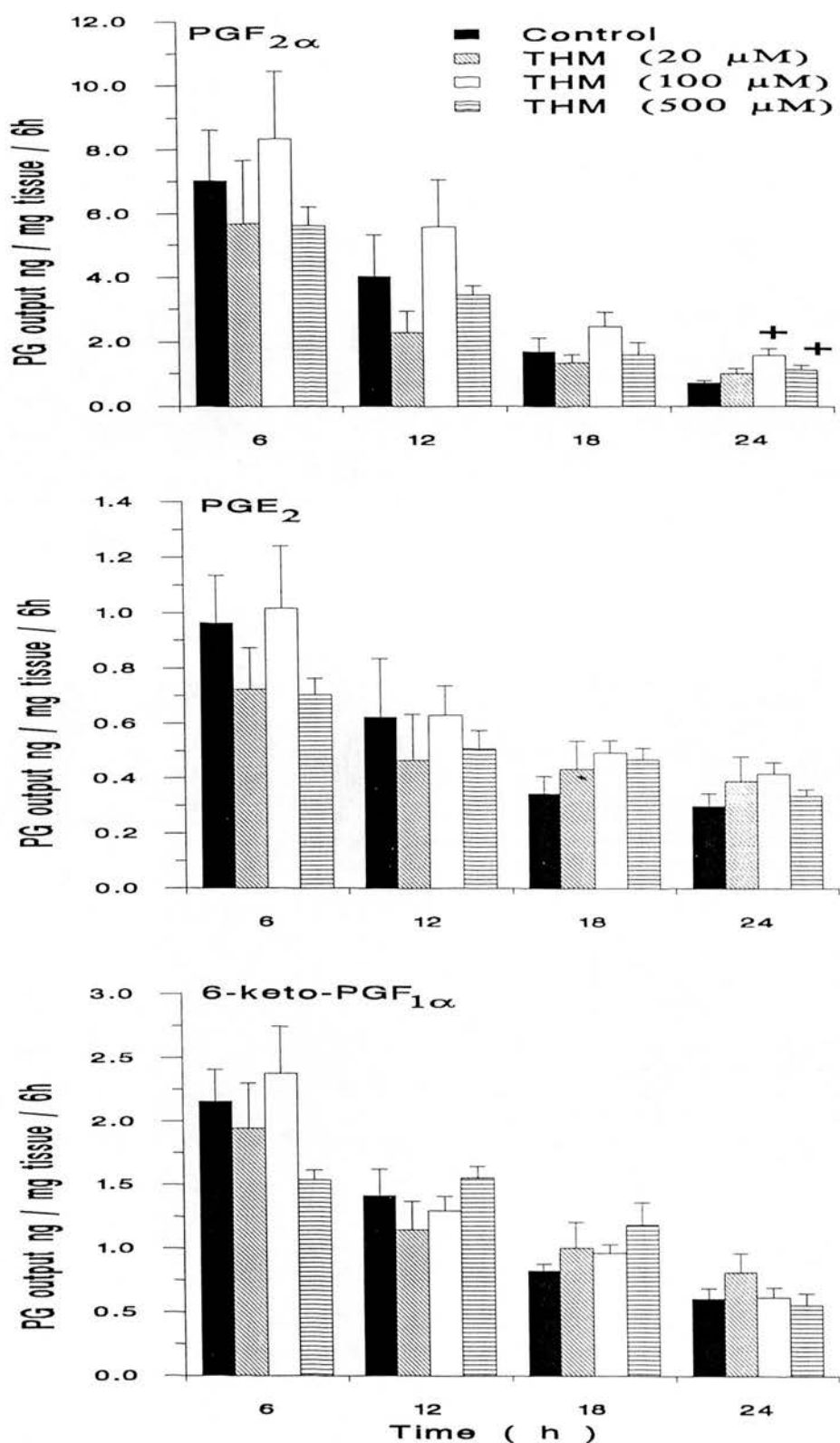
*PGE<sub>2</sub> output* - PGE<sub>2</sub> output was unaffected by either THM or ARA treatment throughout culture.

*6-keto-PGF<sub>1α</sub> output* - 6-keto-PGF<sub>1α</sub> output was unaffected by either THM or ARA treatment throughout culture.

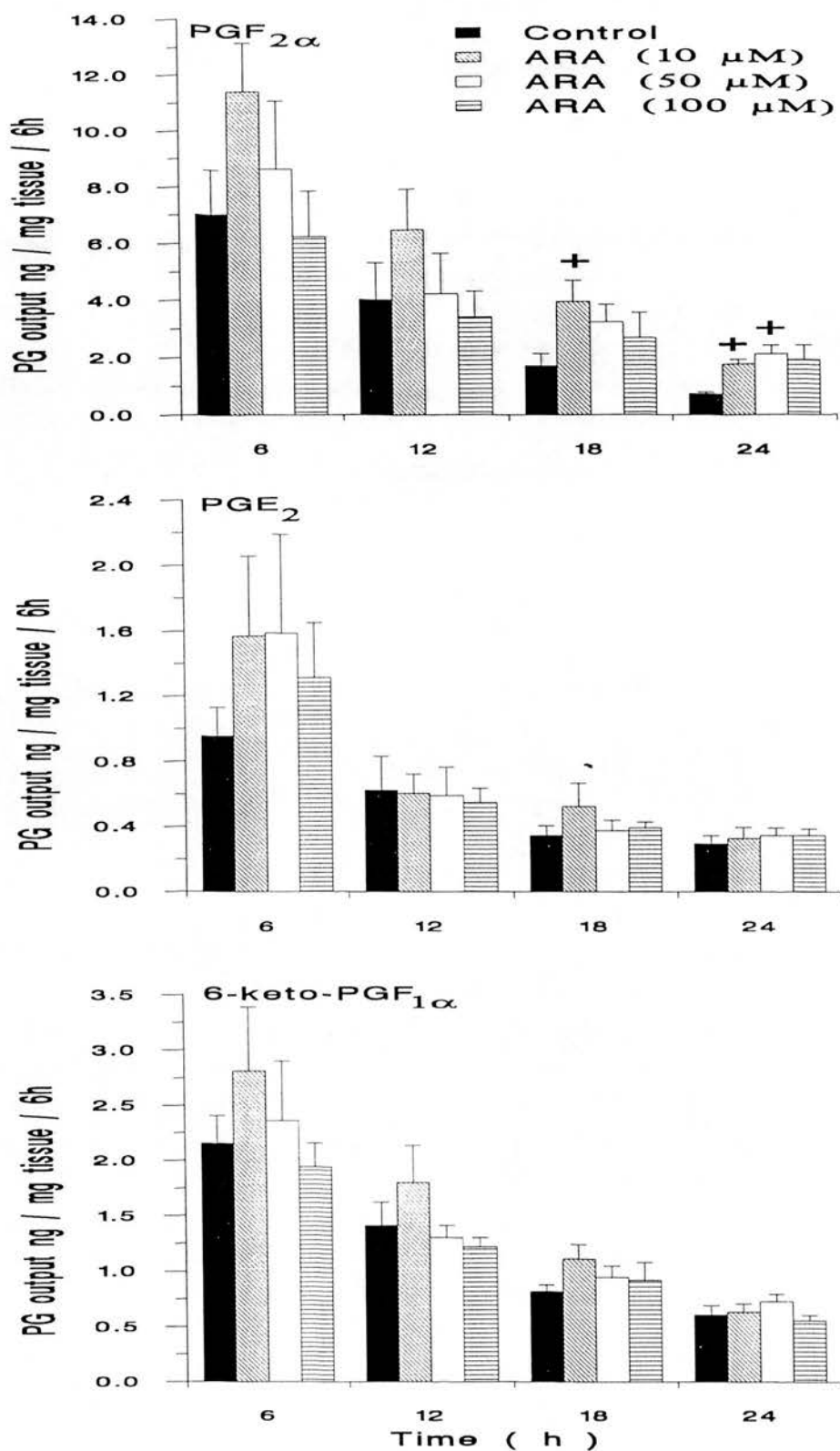
### **Discussion**

THM treatment had no effect on PGF<sub>2α</sub> output from the day 22 pregnant chorio-allantoic placenta, but did result in slight increases in PGF<sub>2α</sub> output from the chorio-allantoic placenta of day 29 and 36 pregnant guinea-pigs but only after 24 h of culture. PGE<sub>2</sub> and 6-keto- PGF<sub>1α</sub> outputs were increased by THM following 18 and 24 h of culture in day 22 and 29 pregnant animals. However, neither PG was affected by THM on day 36 of pregnancy.

Inhibition of PLA<sub>2</sub> with ARA had very little effect on the chorio-allantoic placenta in comparison to its dramatic effects on the endometrium. ARA only slightly inhibited PGF<sub>2α</sub> and PGE<sub>2</sub> output on day 22 of pregnancy, had no effect on either of the two PGs on day 29 of pregnancy, and increased PGF<sub>2α</sub> output after 18 and 24 h of culture from day 36 pregnant animals. 6-keto-PGF<sub>1α</sub> output was also increased following the inhibition of PLA<sub>2</sub> with ARA in both day 22 and 29 pregnant animals, but was unaffected on day 36 of pregnancy. Therefore, PLA<sub>2</sub> would not appear to have an important role in the control of PG synthesis from the chorio-allantoic placenta. It is particularly unexpected that ARA treatment actually increased PGF<sub>2α</sub> output from the fetal



**Figure 58.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the chorio-allantoic placenta of day 36 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500 μM). + Significantly ( $P < 0.05$ ) higher than the control value for the same PG at the same time.



**Figure 59.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the chorio-allantoic placenta of day 36 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM). + Significantly ( $P < 0.05$ ) higher than the control value for the same PG at the same time.

placenta after 18 and 24 h of culture in day 36 pregnant animals, especially since relatively high concentrations of  $\text{PGF}_{2\alpha}$  are produced by the chorio-allantoic placenta at that time.

## **E. Effects of THM and ARA treatment on PG output from the fetal membranes**

### **Results**

#### **i. Fetal membranes from day 29 pregnant animals**

The effects of THM and ARA treatment on PG outputs from the fetal membranes of day 29 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 60 and 61.

*PGF<sub>2α</sub> output* - No significant changes in  $\text{PGF}_{2\alpha}$  output were measured following THM treatment, except with 100  $\mu\text{M}$  THM which significantly ( $P < 0.05$ ) increased  $\text{PGF}_{2\alpha}$  output at 24 h of culture. ARA treatment tended to inhibit  $\text{PGF}_{2\alpha}$  output, with significant ( $P < 0.05$ ) decreases measured following 10  $\mu\text{M}$  ARA treatment at 6 and 24 h, following 50  $\mu\text{M}$  ARA treatment at 24 h, and following 100  $\mu\text{M}$  ARA treatment at 6 and 24 h of culture.

*PGE<sub>2</sub> output* -  $\text{PGE}_2$  output was generally unaffected following THM or ARA treatment, except with 20 and 100  $\mu\text{M}$  THM at 24 h which both significantly increased  $\text{PGE}_2$  output ( $P < 0.05$ ).

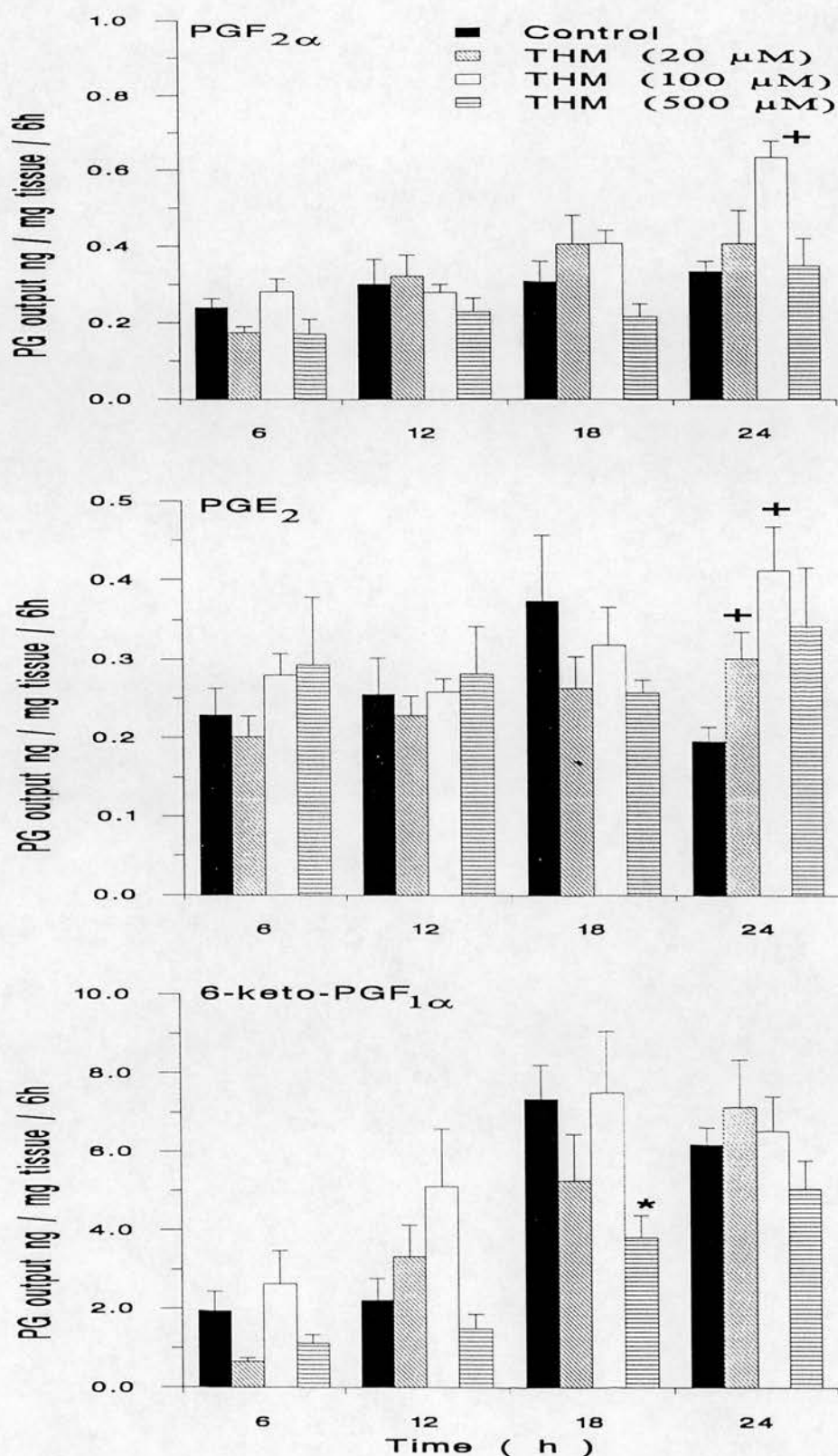
*6-keto-PGF<sub>1α</sub> output* - The output of 6-keto- $\text{PGF}_{1\alpha}$  was significantly ( $P < 0.05$ ) decreased with 500  $\mu\text{M}$  THM at 24 h and with 10  $\mu\text{M}$  ARA at 6 h of culture. No other significant changes in 6-keto- $\text{PGF}_{1\alpha}$  output were measured.

#### **ii. Fetal membranes from day 36 pregnant animals**

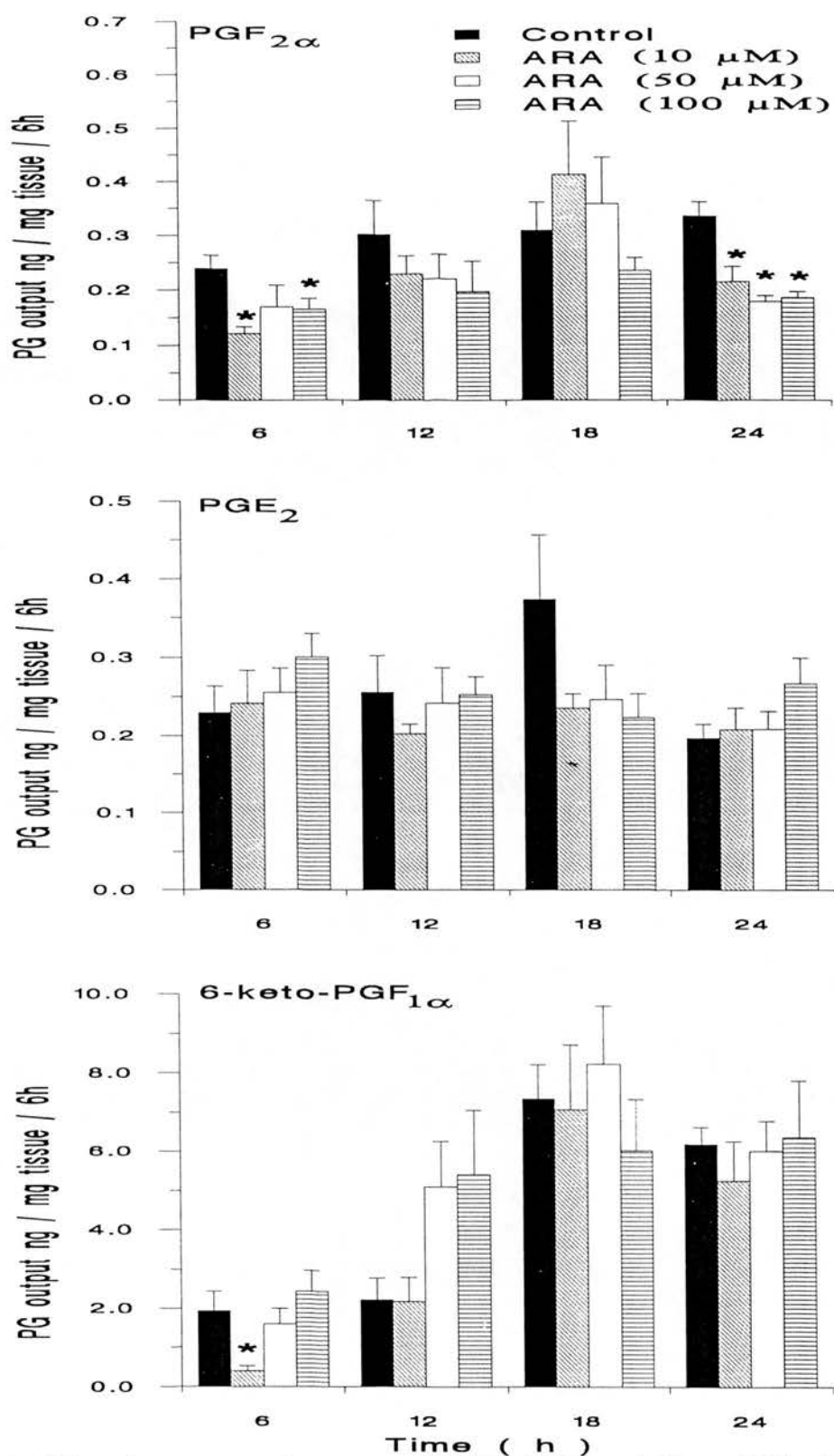
The effects of THM and ARA treatment on PG outputs from the fetal membranes of day 36 pregnant guinea-pigs during 24 h tissue culture and sampled every 6 h are illustrated in Figures 62 and 63.

*PGF<sub>2α</sub> output* - The output of  $\text{PGF}_{2\alpha}$  appeared to be unaffected by THM treatment, except for a significant ( $P < 0.05$ ) potentiation with 100  $\mu\text{M}$  THM at 24 h of culture. ARA treatment tended to decrease  $\text{PGF}_{2\alpha}$  output; however, this decrease was not to a significant level.

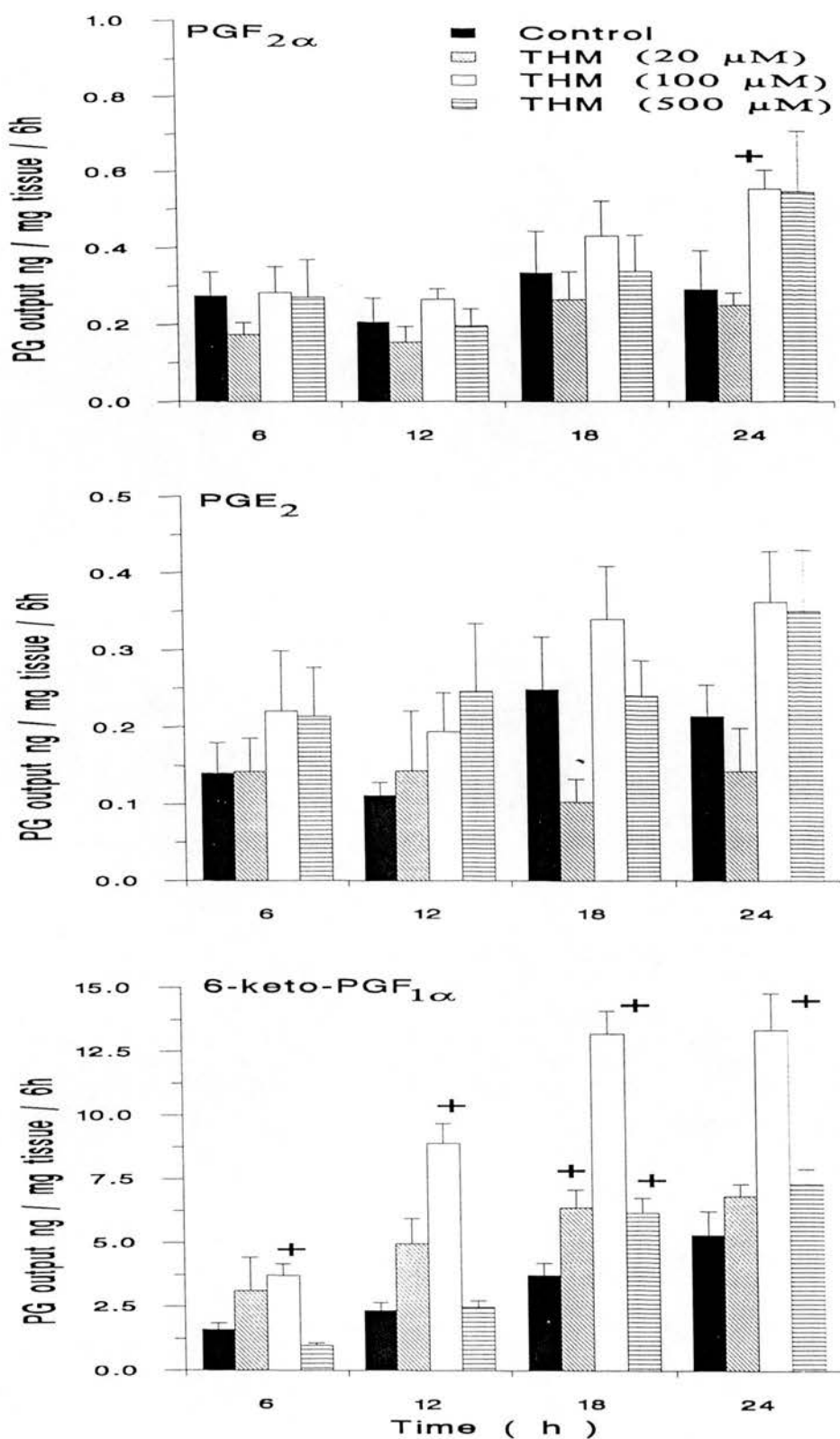




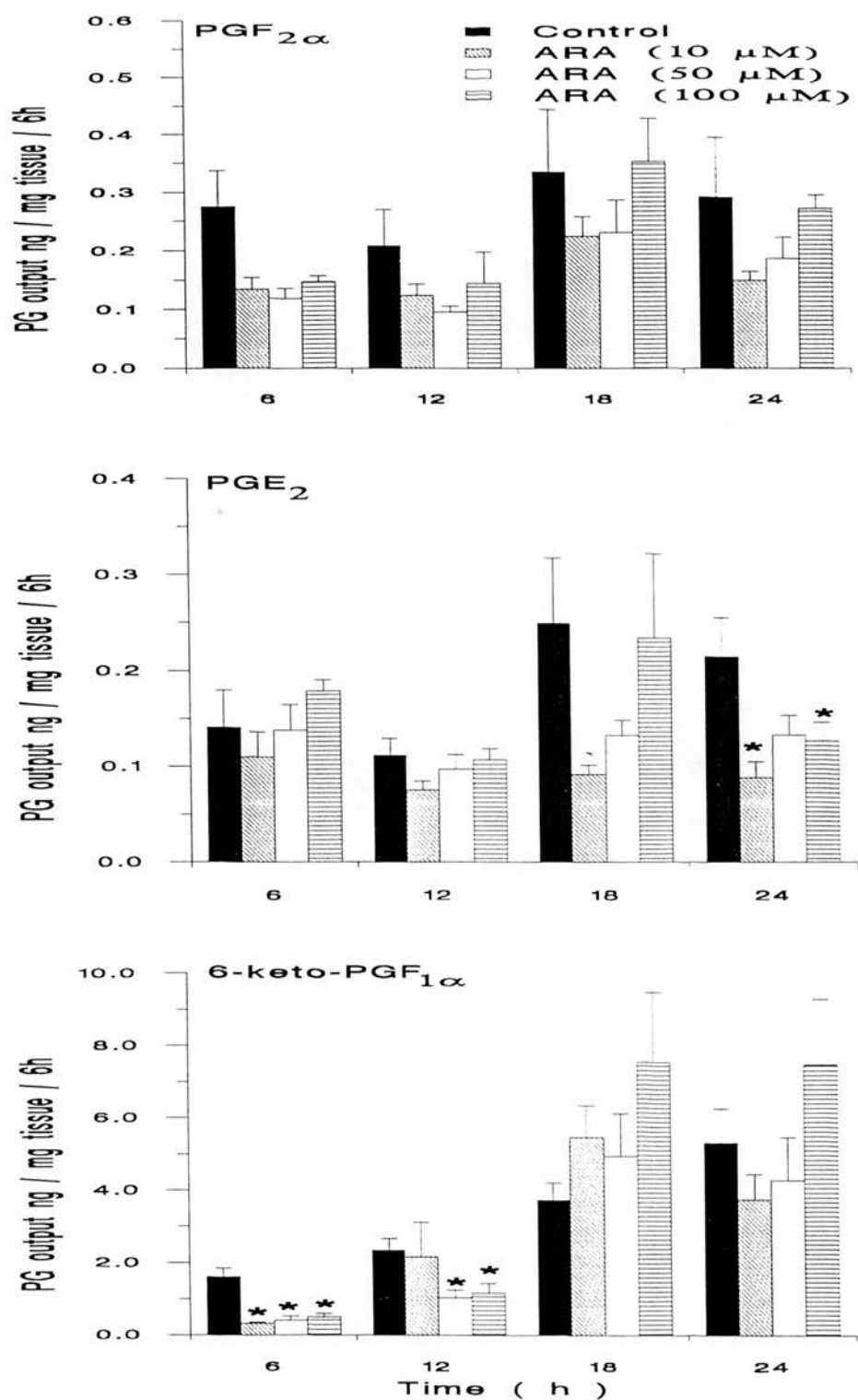
**Figure 60.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the fetal membranes of day 29 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500 μM). Significantly ( $P < 0.05$ ) lower (\*) or higher (+) than the control value for the same PG at the same time.



**Figure 61.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the fetal membranes of day 29 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM).  
 \* Significantly ( $P < 0.05$ ) lower than the control value for the same PG at the same time.



**Figure 62.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the fetal membranes of day 36 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of THM (20, 100 and 500 μM).  
 + Significantly ( $P < 0.05$ ) higher than the control value for the same PG at the same time.



**Figure 63.** Mean ( $\pm$  s.e.m.,  $n=5$ ) outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the fetal membranes of day 36 pregnant guinea-pigs cultured for 24 h and sampled every 6 h, in the absence (control) and presence of ARA (10, 50 and 100 μM).  
 \* Significantly ( $P < 0.05$ ) lower than the control value for the same PG at the same time.

*PGE<sub>2</sub> output* - No significant changes in PGE<sub>2</sub> output were measured following either THM or ARA treatment, except for a significant ( $P < 0.05$ ) decrease in PGE<sub>2</sub> output at 24 h of culture following 10  $\mu$ M and 100  $\mu$ M ARA treatment.

*6-keto-PGF<sub>1 $\alpha$</sub>  output* - The output of 6-keto-PGF<sub>1 $\alpha$</sub>  was significantly ( $P < 0.05$ ) increased by 20  $\mu$ M THM at 18 h, by 100  $\mu$ M THM at 6, 12, 18 and 24 h, and by 500  $\mu$ M THM at 18 h of culture. ARA treatment significantly ( $P < 0.05$ ) decreased 6-keto-PGF<sub>1 $\alpha$</sub>  output; at 6 h with 10, 50 and 100  $\mu$ M ARA, and at 12 h of culture with 50 and 100  $\mu$ M ARA.

## **Discussion**

THM treatment increased PGF<sub>2 $\alpha$</sub>  output, but only after 24 h of culture in both day 29 and 36 pregnant guinea-pig fetal membranes (chorion and amnion). PGE<sub>2</sub> output was also increased in day 29 animals after 24 h of culture. 6-keto-PGF<sub>1 $\alpha$</sub>  output was slightly decreased on day 29, but greatly increased throughout culture in day 36 pregnant animals. ARA treatment generally decreased PGF<sub>2 $\alpha$</sub>  output from the fetal membranes on both days of pregnancy. PGE<sub>2</sub> output was unaffected by ARA treatment on day 29 of pregnancy, and slightly reduced in day 36 pregnant animals. The output of 6-keto-PGF<sub>1 $\alpha$</sub>  was decreased by ARA early during culture, but was unaffected after 18 and 24 h of culture. Since PLA<sub>2</sub> activity was detected in the fetal membranes of day 29 and 36 pregnant animals in section 3:3, the evidence suggests that PLA<sub>2</sub> is involved in the control of PG synthesis in the fetal membranes.

The inhibition of arachidonic acid reacylation appeared to increase PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> output after 24 h of culture. Interestingly, the output of 6-keto-PGF<sub>1 $\alpha$</sub>  was greatly increased by THM in day 36 pregnant animals. Thus, the control of arachidonic acid reacylation appears to be involved in the regulation of PG output, particularly 6-keto-PGF<sub>1 $\alpha$</sub> , from the fetal membranes of the day 36 pregnant guinea-pig.

## Overall Discussion

With regard to the control of arachidonic acid turnover and the resultant PG outputs, it would appear that the control of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs differs amongst guinea-pig uterine and conceptual tissues and at the various stages of pregnancy investigated.

THM, an inhibitor of arachidonic acid reacylation, decreased both  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs and increased 6-keto- $\text{PGF}_{1\alpha}$  output from the endometrium on all days of pregnancy. It would therefore appear that by inhibiting ACS and ACLS activity, the concentration of free arachidonic acid remained high enabling increased 6-keto- $\text{PGF}_{1\alpha}$  output. However,  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs were reduced. This may be explained by the theory that by inhibiting general arachidonic acid reacylation, the appropriate phospholipids (i.e. containing *sn*-2 position arachidonic acid) available to  $\text{PLA}_2$  became limiting. These results suggest that the control of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  synthesis and that of  $\text{PGI}_2$  (as detected by 6-keto- $\text{PGF}_{1\alpha}$ ) are different. This is in agreement with the findings of Poyser (1991) which implicated that a different pool of arachidonic acid and a  $\text{PLA}_2$  enzyme located in a different site are used for the production of 6-keto- $\text{PGF}_{1\alpha}$  than those which are used for the production of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  in the guinea-pig endometrium. Previous studies have also demonstrated that the syntheses of  $\text{PGF}_{2\alpha}$  and  $\text{PGI}_2$  are controlled by different intracellular processes (Riley and Poyser, 1987; 1990). In contrast to the endometrium, in the subplacenta, THM treatment did increase  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs suggesting a role for ACS and ACLS in the control of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs from this tissue. 6-keto- $\text{PGF}_{1\alpha}$  output was unaffected by THM in the subplacenta, strengthening the theory that the syntheses of these prostaglandins are controlled differently. In the chorio-allantoic placenta,  $\text{PGF}_{2\alpha}$  output was unaffected by THM on day 22 of pregnancy and increased slightly on days 29 and 36 of pregnancy, but only following 24 h of culture. The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were also increased by THM on days 22 and 29 (but not day 36) of pregnancy after 18 and 24 h of culture. Therefore, the control of arachidonic acid reacylation does not appear to have a major role in the control of PG synthesis from the chorio-allantoic placenta. Similarly in the

fetal membranes (chorion and amnion), THM increased the outputs of  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  but only after 24 h of culture. However, the output of 6-keto- $\text{PGF}_{1\alpha}$  was greatly increased by THM on day 36 of pregnancy, suggesting a crucial role for arachidonic acid reacylation in the control of 6-keto- $\text{PGF}_{1\alpha}$  synthesis, in particular towards mid-pregnancy in the guinea-pig fetal membranes.

Evidently,  $\text{PLA}_2$  has a crucial role in the regulation of PG synthesis, not only from the non-pregnant, but also from the pregnant, guinea-pig endometrium. In contrast, the inhibition of  $\text{PLA}_2$  with ARA had very little effect on PG output from both the subplacenta and chorio-allantoic placenta. ARA did decrease  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs slightly from day 22 pregnant chorio-allantoic placental tissue, but unexpectedly increased  $\text{PGF}_{2\alpha}$  output after 18 and 24 h of culture on day 36 of pregnancy, and 6-keto- $\text{PGF}_{1\alpha}$  output on days 22 and 29 of pregnancy (at 6 and 12 h of culture). Thus,  $\text{PLA}_2$  does not appear to be vital to the regulation of PG output from the subplacenta or chorio-allantoic placenta in the guinea-pig. However, in the fetal membranes, ARA did decrease  $\text{PGF}_{2\alpha}$  output in both day 29 and 36 pregnant animals,  $\text{PGE}_2$  output in day 36 pregnant animals and 6-keto- $\text{PGF}_{1\alpha}$  output early during culture (6-keto- $\text{PGF}_{1\alpha}$  output was unaffected after 18 and 24 h of culture), suggesting a role for  $\text{PLA}_2$  in the control of PG synthesis from the fetal membranes. The fact that 6-keto- $\text{PGF}_{1\alpha}$  output was unaffected by ARA treatment after 18 and 24 h of culture, would again suggest an alternative route for 6-keto- $\text{PGF}_{1\alpha}$  synthesis in guinea-pig conceptual tissues.



**3:6     Effects of calcium ionophore A23187 on thimerosal, p-hydroxymercuribenzoic acid and aristolochic acid inhibited prostaglandin output from day 7 non-pregnant guinea-pig uterus superfused *in vitro*.**

**Introduction**

From the previous experiments, it is clear that the control of arachidonic acid turnover plays an important role in the regulation of PG production by both uterine and conceptual tissues in the guinea-pig. However, the tissue culture experiments investigated the effects of inhibitors of arachidonic acid turnover during a 24 h period. These conditions produced unclear results since, although the inhibition of acyl-CoA synthetase (ACS) and acyl-CoA:lysophospholipid acyltransferase (ACLS) may increase the concentration of free arachidonic acid available for PG synthesis and therefore increase PG output over a short time, during a longer time period (such as a 24 h tissue culture), the inhibition of ACS and ACLS may block the entire arachidonic acid turnover system and result in a decrease in the amount of arachidonic acid incorporated at the appropriate *sn*-2 position in phospholipids suitable for PLA<sub>2</sub> action. This would reduce the amount of available free arachidonic acid and thereby decrease PG synthesis and output.

It is considered that activation of PLA<sub>2</sub> by calcium (Downing and Poyser, 1983), which leads to the release of free arachidonic acid from PC and PE (Ning and Poyser, 1984), is necessary for the stimulation of arachidonic acid release and the initiation of PG synthesis in the guinea-pig endometrium. The calcium ionophore, A23187, stimulates uterine PG synthesis by a mechanism dependent upon the presence of extracellular calcium (Poyser, 1984b). Consequently, the effects of THM, HMB and ARA on PG output and on the stimulatory actions of A23187 from the day 7 non-pregnant guinea-pig uterus superfused *in vitro* have been investigated.

**Methods**

The uteri from fifteen day 7 guinea-pigs were removed and separated into the two horns. Each uterine horn was weighed, "opened" by a longitudinal incision, and

superfused with Krebs solution, aerated with 5 % CO<sub>2</sub> and 95 % O<sub>2</sub>, at 37°C at a rate of 5 ml/min. Each horn was superfused initially for a “settling period” of 60 min and the samples of superfusate were collected for 10-min periods over the next 100 min (i.e. 10 samples/uterine horn).

Expt. 1 - A23187 (1 µg/ml) was present in the Krebs solution superfusing both uterine horns from 5 guinea-pigs during the collection of samples 6 and 7. In addition, 100 µM thimerosal (THM) was present in the Krebs solution superfusing one uterine horn from each guinea-pig during the collection of samples 4-7.

Expt. 2 - A23187 (1 µg/ml) was present in the Krebs solution superfusing both uterine horns from 5 guinea-pigs during the collection of samples 6 and 7. In addition, 100 µM p-hydroxymercuribenzoic acid (HMB) was present in the Krebs solution superfusing one uterine horn from each guinea-pig during the collection of samples 4-7.

Expt. 3 - A23187 (1 µg/ml) was present in the Krebs solution superfusing both uterine horns from 5 guinea-pigs during the collection of samples 6 and 7. In addition, 100 µM aristolochic acid (ARA) was present in the Krebs solution superfusing one uterine horn from each guinea-pig during the collection of samples 4-7.

A concentration of 100 µM for each inhibitor was used since this concentration had resulted in measurable changes in PG output from guinea-pig uterine tissue in previous experiments. After collection, PGs were solvent-extracted from the samples of superfusate and were stored in 10 ml ethyl acetate at -20°C. The amounts of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> present in the extracts were measured by radioimmunoassay. The outputs of PGs were calculated per min per 100 mg wet weight of uterus.

### **Statistical tests**

Changes in the output of PGs with time were analysed by Duncan's multiple range test. Other comparisons were made by Student's t-test, or if the variances of the two groups were significantly different by the variance ratio F test, by the modified t-test if the variances were unequal.

## Results

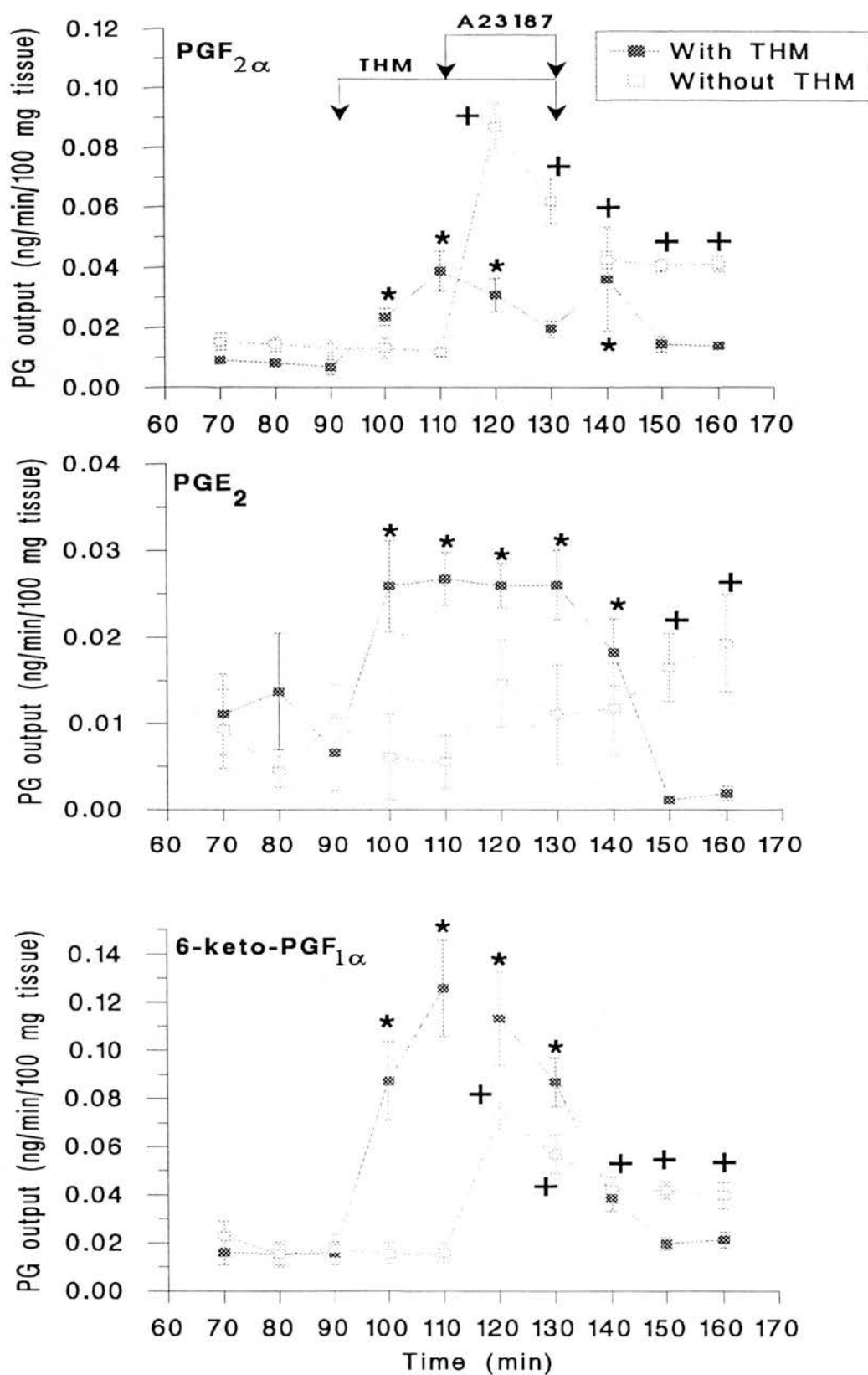
The effects of THM, HMB and ARA on the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  from the day 7 superfused guinea-pig uterus are shown in Figures 64, 65 and 66, respectively.

In all control experiments (i.e. superfused uteri not treated with either THM, HMB or ARA), A23187 significantly ( $P < 0.05$ ) increased the outputs of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$ . These increased outputs in  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  were maintained throughout the experiment. The output of  $\text{PGE}_2$  was generally unaffected by A23187 treatment.

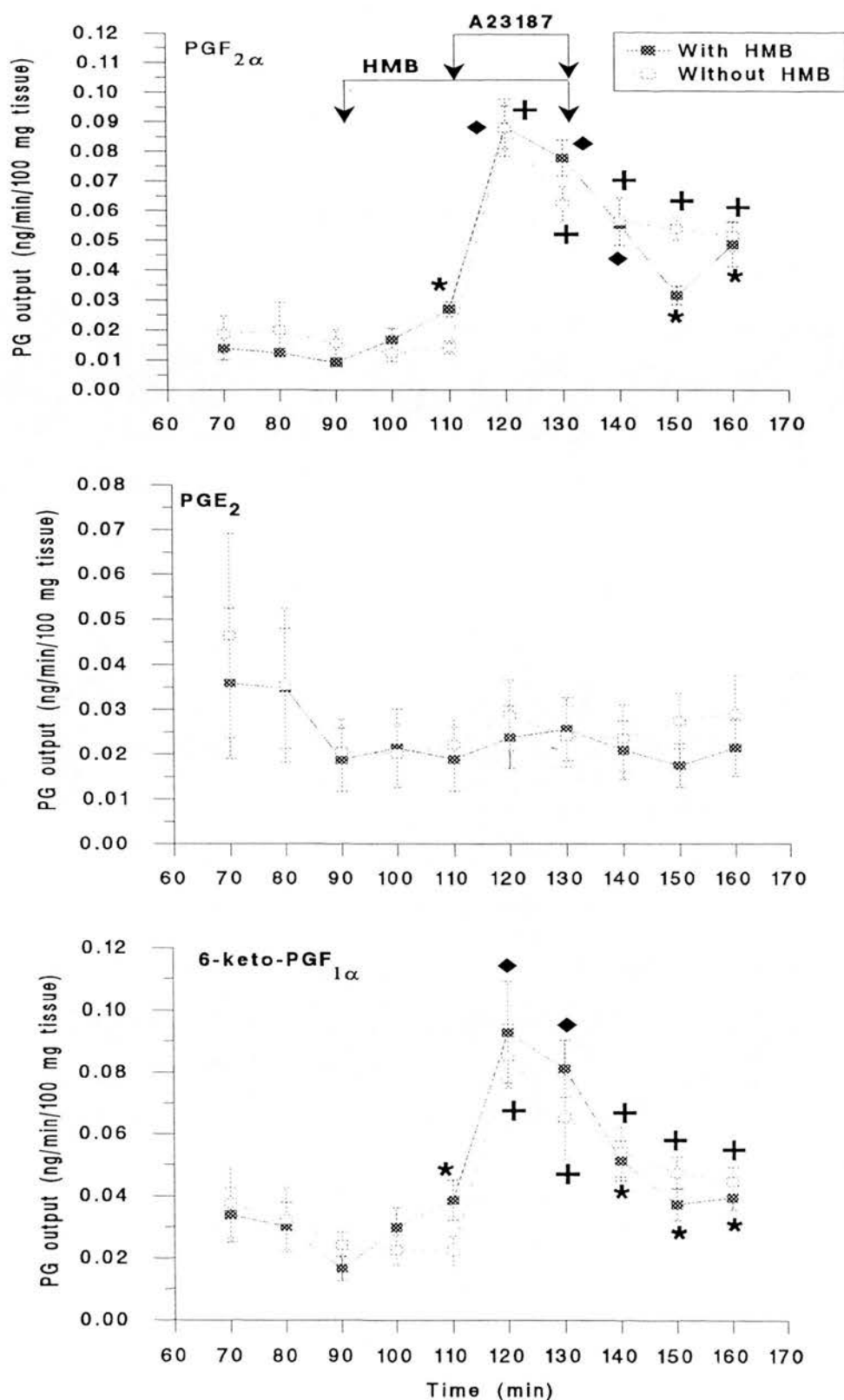
Expt. 1 - THM significantly ( $P < 0.05$ ) increased the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  5.7-, 4.0- and 8.0-fold, respectively from the day 7 superfused uterus. In the presence of THM, A23187 treatment failed to further stimulate the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$ . In fact, following A23187 treatment, the output of  $\text{PGF}_{2\alpha}$  was significantly lower from THM treated uterus compared to control uterus. However, 6-keto- $\text{PGF}_{1\alpha}$  output was significantly greater from THM treated uterus than from control uterus even during A23187 treatment.

Expt. 2 - HMB significantly ( $P < 0.05$ ) increased the outputs of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  2.9- and 2.3-fold, respectively from the day 7 superfused uterus. Treatment with A23187 in the presence of HMB further stimulated ( $P < 0.05$ ) the outputs of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  3.3- and 2.4-fold, respectively. Following A23187 treatment, the outputs of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  were similar in both HMB treated and untreated uteri. HMB and/or A23187 treatment had no effect on the output of  $\text{PGE}_2$ .

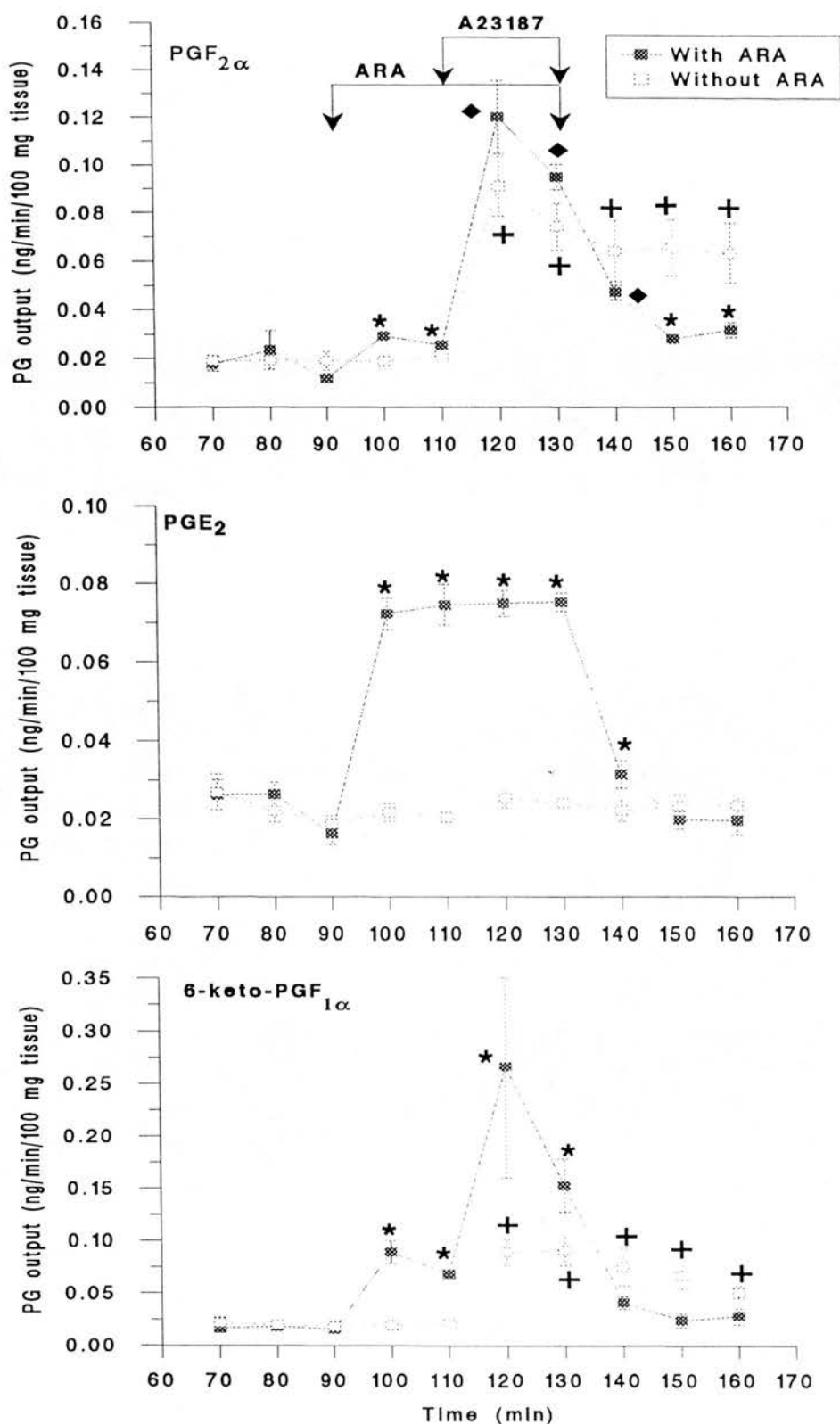
Expt. 3 - ARA significantly ( $P < 0.05$ ) increased the outputs of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  2.5-, 4.6- and 5.7-fold, respectively from the day 7 superfused uterus. Treatment with A23187 in the presence of ARA further stimulated ( $P < 0.05$ ) the outputs of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  4.7- and 3.9-fold, respectively. A23187 treatment had no effect on  $\text{PGE}_2$  output from either ARA treated or untreated uteri.



**Figure 64.** Effect of THM (100  $\mu$ M) on mean ( $\pm$  s.e.m.,  $n=5$ ) A23187-stimulated outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> from the day 7 guinea-pig uterus superfused *in vitro*. \* Significantly ( $P < 0.05$ ) higher than before any treatment. + Significantly higher than before A23187 treatment alone.



**Figure 65.** Effect of HMB (100  $\mu$ M) on mean ( $\pm$  s.e.m.,  $n=5$ ) A23187-stimulated outputs of PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  from the day 7 guinea-pig uterus superfused *in vitro*. \* Significantly ( $P < 0.05$ ) higher than before any treatment. + Significantly higher than before A23187 treatment alone. ♦ Significantly ( $P < 0.05$ ) higher than before any treatment and with HMB treatment alone.



**Figure 66.** Effect of ARA (100  $\mu$ M) on mean ( $\pm$  s.e.m.,  $n=5$ ) A23187-stimulated outputs of PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  from the day 7 guinea-pig uterus superfused *in vitro*. \* Significantly ( $P < 0.05$ ) higher than before any treatment. + Significantly higher than before A23187 treatment alone. ♦ Significantly ( $P < 0.05$ ) higher than before any treatment and with HMB treatment alone.

## Discussion

All three inhibitors, namely THM, HMB and ARA, significantly increased the outputs of  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  from the day 7 superfused guinea-pig uterus.  $\text{PGE}_2$  output was also significantly increased by THM and ARA, but not by HMB, treatment. THM and HMB both inhibit acyl-CoA synthetase and acyl-CoA:lysophospholipid acyltransferase and therefore inhibit arachidonic acid reacylation. THM and HMB do not affect  $\text{PLA}_2$  activity or the activity of the enzymes involved in PG synthesis (Hunter *et al.*, 1984; Goppelt-Streube *et al.*, 1986). Consequently, the amount of free arachidonic acid available for PG synthesis may actually be increased over a short time period (such as in these short superfusion experiments), since free arachidonic acid concentrations are not reincorporated into phospholipids and remain available for PG synthesis resulting in increased PG output from the guinea-pig uterus.

The output of 6-keto- $\text{PGF}_{1\alpha}$  was increased the most by THM treatment, suggesting that the activity of ACS and ACLS are important in the control of 6-keto- $\text{PGF}_{1\alpha}$  synthesis in the guinea-pig uterus. A23187 failed to further stimulate PG synthesis from THM-treated guinea-pig uterus. Since  $\text{PGF}_{2\alpha}$  output was significantly greater from control (no THM) A23187-treated uteri compared to A23187 + THM-treated uteri, it would appear that THM treatment inhibited the stimulatory action of A23187 on  $\text{PGF}_{2\alpha}$  synthesis from the superfused guinea-pig uterus. The outputs of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were increased by a far greater extent following THM treatment (alone) compared to PG outputs stimulated in control A23187-treated uteri, therefore, the addition of A23187 to THM-treated uteri may have failed to release more free arachidonic acid for PG synthesis.

HMB was shown to be a less potent inhibitor of ACS and ACLS than THM, since HMB increased  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs 2.9- and 2.3-fold compared to 5.7- and 8.0-fold, respectively following THM treatment. Also,  $\text{PGE}_2$  output was unaffected by HMB treatment but was increased 4.0-fold by THM treatment. Indeed, in contrast to THM treated uteri, the addition of A23187 to HMB-treated uteri did further stimulate  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs 3.3- and 2.4-fold indicating that arachidonic acid was not limiting.  $\text{PGE}_2$  was unaffected by A23187 indicating that the



control of PGE<sub>2</sub> synthesis in the guinea-pig uterus is probably not controlled by the activation of PLA<sub>2</sub> by calcium.

Unexpectedly, ARA (a PLA<sub>2</sub> inhibitor) treatment did not decrease PG output. This may have been due to the pretreatment time with ARA being too short to inhibit PLA<sub>2</sub>. The reason for the increase in the outputs of PGF<sub>2α</sub> and 6-keto-PGF<sub>1α</sub> following treatment with ARA is unclear. It may be the case that ARA treatment influenced the activities of PGH synthase and/or PGDH. Stimulation of PGH synthase and/or inhibition of PGDH would result in increased PG production. The addition of A23187 further stimulated both PGF<sub>2α</sub> and 6-keto-PGF<sub>1α</sub>. This may be due to A23187 stimulating PLC activity (which releases arachidonic acid from PI) and thus increasing PG synthesis. Indeed, this has been suggested to occur previously in the superfused guinea-pig uterus *in vitro* (Poyser, 1991) since the guinea-pig uterus has a large source of a PLC enzyme specific for PI (Bennett and Crooke, 1987). PGE<sub>2</sub> output was significantly increased during ARA treatment, however this was not sustained. Similarly to the results obtained in experiment 1 and 2, A23187 did not affect PGE<sub>2</sub> output strengthening the evidence that the activation of PLA<sub>2</sub> (or PLC) by calcium is not involved in the control of PGE<sub>2</sub> synthesis from the superfused guinea-pig uterus.

**3:7     Effects of thimerosal, p-hydroxymercuribenzoic acid and aristolochic acid on prostaglandin synthesis by homogenates of day 7 non-pregnant guinea-pig endometrium.**

**Introduction**

The previous experiment investigated the effect of inhibitors of arachidonic acid turnover and the stimulatory effect of A23187 on PG synthesis in the presence of the inhibitors in day 7 guinea-pig uterus. The following experiment again investigated the effects of inhibitors of arachidonic acid turnover in day 7 animals, but during a 1 h incubation of uterine homogenates in the presence of free arachidonic acid i.e. arachidonic acid was not limiting for PG synthesis.

**Methods**

Three virgin guinea-pigs that had exhibited at least three normal oestrous cycles were killed by stunning and incision of the neck on day 7 of the cycle. The uterus was removed and separated into its two horns. Each uterine horn was "opened" by a longitudinal incision and the endometrium dissected away from the myometrium by cutting away 1-2 mm<sup>3</sup> sections of endometrium using a pair of fine scissors. The endometrial tissue from each animal was divided into four parts as equally as possible, blotted dry, and each part weighed. Each part was then homogenised using a ground glass homogeniser in 10 ml ice-cold Krebs solution containing 2 µg/ml arachidonic acid and either no inhibitor (control), 100 µM HMB, 100 µM THM or 100 µM ARA. The time interval between removal of the uterus and completion of homogenisation was never greater than 15 min. The homogenates were then incubated at 37°C for 60 min in a shaking water bath. After incubation, the pH of the incubates were lowered to 4.0 by addition of HCl and the PGs were extracted by shaking twice with 15 ml redistilled ethyl acetate. The two ethyl acetate extracts obtained from each incubate were combined and evaporated to dryness at 45°C on a rotary evaporator. Each extract was then redissolved in 10 ml redistilled ethyl acetate and stored at -20°C. The amounts of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> present in the extracts were later measured by radioimmunoassay (see section 2:5).

## Statistical Tests

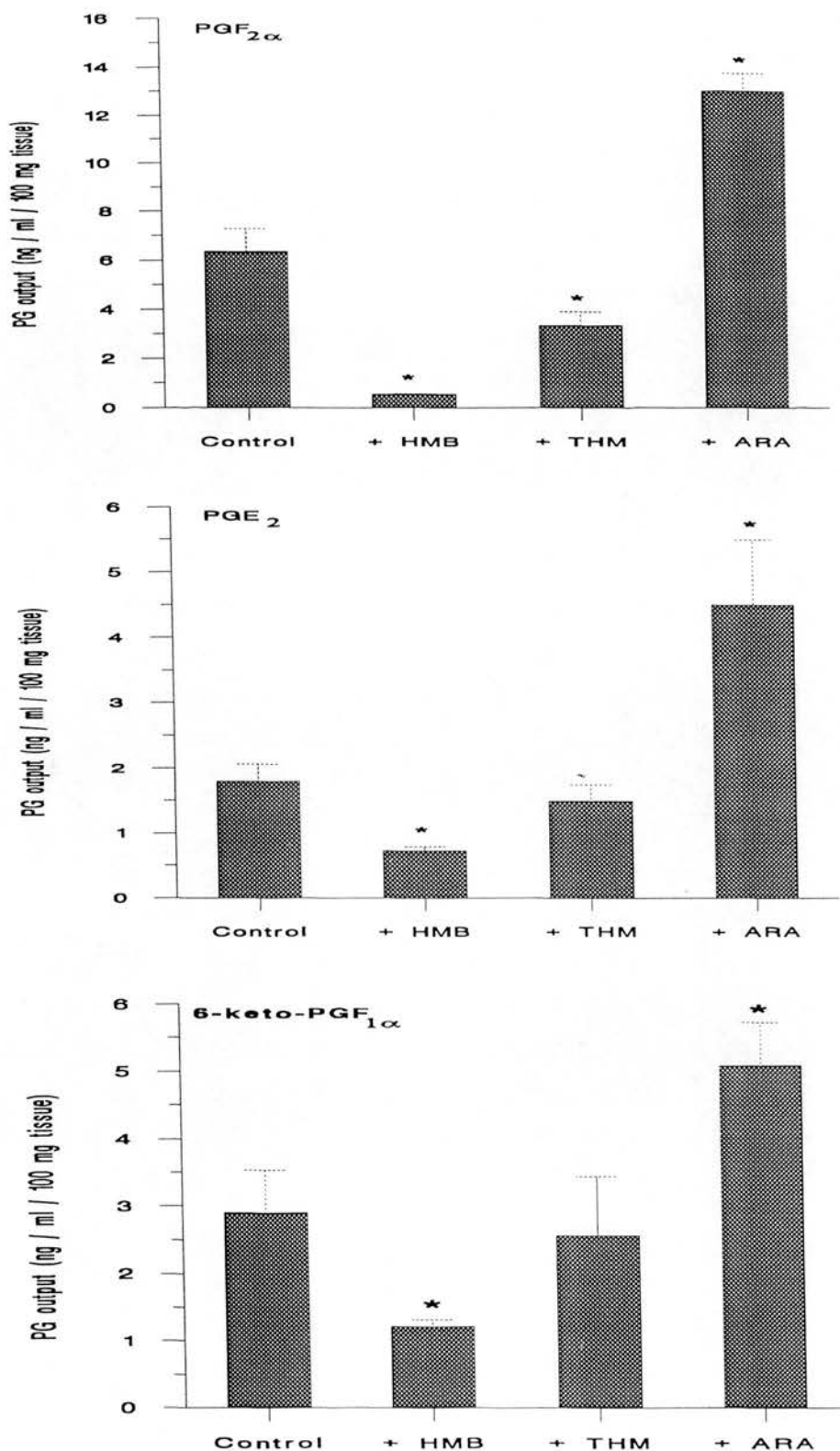
Results were analysed by the Student's t-test or, if the variances of the two groups were significantly different by the variance ratio F test, by a modified t-test for unequal variances.

## Results

The effects of THM, HMB and ARA treatment on the amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesized by homogenates of endometrium from day 7 guinea-pigs are illustrated in Figure 67. The amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesized by control (untreated) homogenates of guinea-pig endometrium incubated for 1 h at  $37^\circ\text{C}$  were  $6.36 \pm 0.92$ ,  $1.80 \pm 0.27$  and  $2.90 \pm 0.64$  ng/ml/100 mg tissue, respectively. THM treatment significantly ( $P < 0.05$ ) reduced  $\text{PGF}_{2\alpha}$  synthesis, but  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  syntheses were unaffected. HMB treatment also significantly reduced  $\text{PGF}_{2\alpha}$  synthesis ( $P < 0.005$ ), and also significantly decreased the syntheses of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  ( $P < 0.05$ ). ARA treatment significantly ( $P < 0.05$ ) increased the amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  synthesized by 2.0-, 2.5- and 1.8-fold, respectively.

## Discussion

The amounts of  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  formed in the control homogenates represent PG synthesis during a 1 h incubation period in the presence of at least  $2 \mu\text{g/ml}$  free arachidonic acid (arachidonic acid is also released from the tissue during the homogenisation process, Mitchell, Poyser and Wilson, 1977). No inhibitors were used in the control, thus normal arachidonic acid turnover could be assumed to be occurring i.e. ACS and ACLS reacylated free arachidonic acid, whilst  $\text{PLA}_2$  deacylated arachidonic acid for the purpose of PG synthesis. The presence of THM (a potent arachidonic acid reacylation inhibitor) did not affect either  $\text{PGE}_2$  or 6-keto- $\text{PGF}_{1\alpha}$  synthesis, suggesting that the free arachidonic acid was not reacylated and used for the synthesis of these two PGs in similar amounts to those synthesized by control homogenates. However, THM treatment did significantly decrease  $\text{PGF}_{2\alpha}$  synthesis.



**Figure 67.** Mean ( $\pm$  s.e.m.,  $n=3$ ) *in vitro* PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> production by homogenates of uterine tissues from day 7 non-pregnant guinea-pigs in the absence (control) and presence of HMB (+HMB), THM (+THM) and ARA (+ARA).

\* Significantly ( $P < 0.05$ ) different to control value for the same PG.

This possibly resulted from the reduction in appropriate phospholipids available to PLA<sub>2</sub> (arachidonic acid present in *sn*-2 position of PC and PE) due to the inhibition of arachidonic acid turnover. Treatment with HMB significantly reduced the synthesis of all three PGs. This may have been due to its weak inhibitory effect on ACS and ACLS activity, and therefore, free arachidonic acid continued to be reacylated, together with a slight reduction in appropriate phospholipids for the release of arachidonic acid by PLA<sub>2</sub> for PG synthesis. Both THM and HMB do not affect PLA<sub>2</sub> activity or the activity of enzymes involved in PG synthesis (Hunter *et al.*, 1984; Goppelt-Streube *et al.*, 1986), thus the inhibition of PG synthesis by guinea-pig uterine homogenates cannot be due to either of those factors.

The significant increase in all three PGs following ARA treatment is somewhat surprising. It was expected that free arachidonic acid would be reacylated (since no THM or HMB were present) and PLA<sub>2</sub> inhibited, therefore resulting in reduced PG synthesis. However, increased PG synthesis resulted. A possible explanation for these findings, is that the inhibition of PLA<sub>2</sub> resulted in the “switching on” of PLC, thus releasing free arachidonic acid from PI for the purpose of PG synthesis. It should also be considered that ARA may not inhibit PLA<sub>2</sub> activity quickly. Indeed, ARA treatment did not decrease PG output from the uterus during short 40 min treatments in superfusion experiments (section 3.6), but did decrease PG outputs during long term treatment of endometrium in 24 h tissue culture experiments (section 3.4).

Also, during the homogenisation process, which disrupts cell structure, free arachidonic acid is known to be released (Mitchell *et al.*, 1977). It is possible that ARA treatment may have little effect on PG synthesis during a short 1 h incubation period since free arachidonic acid was released during homogenisation, and 2 µg/ml arachidonic acid had also been added to the incubates. However, if this were the case, PG synthesis similar to that observed in the control incubates would have been expected. This was not the case. ARA treatment actually increased all three PG syntheses significantly above that seen in control incubates. This would suggest that ARA was having some other effect on PG synthesis. ARA treatment was also observed to significantly increase PGE<sub>2</sub> (but not PGF<sub>2α</sub> or 6-keto-PGF<sub>1α</sub>) synthesis in the subplacenta of day 22 pregnant guinea-pigs during 24 h tissue culture (see Figure

49) and PGE<sub>2</sub> synthesis from day 7 non-pregnant guinea-pig uterus superfused *in vitro* (see Figure 66). Neither PGF<sub>2α</sub> or 6-keto-PGF<sub>1α</sub> outputs were increased to such an extent as that of PGE<sub>2</sub> during superfusion following ARA treatment, however, their outputs were increased by the presence of A23187, whilst that of PGE<sub>2</sub> was not enhanced by A23187 treatment (see Figure 66). Perhaps ARA may influence the activity of other enzymes involved in PG synthesis, such as PGHS. This could explain the observations of increased PG synthesis, significantly greater than that seen in control incubates, in Figure 67. If we assume that normal arachidonic acid turnover and PG synthesis is occurring in the control incubates in normal conditions ie. without the presence of either HMB, THM or ARA, and where arachidonic acid is not limiting since free arachidonic acid is released due to cellular disruption during homogenisation and free exogenous arachidonic acid was also added, we would expect to observe maximum PG synthesis in these incubates. Therefore, since ARA treatment further increased PG synthesis, ARA treatment must have some other effect such as increasing PGHS activity. PGHS exhibits self-catalysed breakdown during the synthesis of PGs (Lands, 1979). Consequently, the total amount of PGs synthesized by endometrial homogenates is indicative of the concentration of PGHS, especially since the metabolism of PGs in the absence of NAD<sup>+</sup> by the guinea-pig uterus is negligible (< 5%) (Poyser, 1979). This would suggest that ARA treatment of guinea-pig endometrial homogenates resulted in an increase in PGHS activity. An increase in PGHS activity and the presence of free exogenous arachidonic acid (ie. arachidonic acid was not limiting) would explain the unexpected increase in PG synthesis following the inhibition of PLA<sub>2</sub> by ARA.

## CHAPTER 4 : GENERAL DISCUSSION

PGF<sub>2α</sub> has been identified as the uterine luteolytic hormone in several mammalian species, including the guinea-pig (Horton and Poyser, 1976; Poyser, 1981). During the oestrous cycle, oestradiol secretion from the ovary increases from day 10 (Joshi *et al.*, 1973) which precedes the increase in PGF<sub>2α</sub> secretion from the uterus by 24 h (Blatchley *et al.*, 1972; Earthy *et al.*, 1975; Antonini *et al.*, 1976). Also, the administration of oestradiol to ovariectomized guinea-pigs maintained on progesterone stimulates uterine PGF<sub>2α</sub> output (Blatchley and Poyser, 1974; Poyser, 1983b). Therefore, oestradiol acting on a progesterone-primed uterus appears to be the physiological stimulus for increased PGF<sub>2α</sub> production by the guinea-pig uterus (particularly by the endometrium) towards the end of the oestrous cycle. Oxytocin is also involved in the regulation of uterine PGF<sub>2α</sub> production in some species such as in the sheep (Lee and Silvia, 1994) but not in the guinea-pig (Donovan, 1961; Poyser and Brydon, 1983; Riley and Poyser, 1987b).

In the guinea-pig, it has been demonstrated that towards the end of the cycle, oestradiol stimulates an increase in the concentration of PGH synthase in the endometrium by inducing increased protein synthesis (Poyser, 1979). However, little is known about how oestradiol and progesterone control endometrial PG synthesis and, in particular, how they control the supply of free arachidonic acid for increased PGF<sub>2α</sub> synthesis at the end of the oestrous cycle.

The rate-limiting step in PG synthesis is generally considered to be the release of free arachidonic acid from lipid stores. In the guinea-pig uterus, 90 % of arachidonic acid is bound to phospholipid, of which 80 % is bound to PC and PE. There is a decrease in the amount of arachidonic acid bound as PC and PE between days 7 and 15 of the cycle (Leaver and Poyser, 1981), suggesting that PC and PE may be the source of arachidonic acid for PG synthesis in the guinea-pig uterus. Further studies demonstrated a specific stimulation of the mechanisms involved in the uptake of arachidonic acid into guinea-pig endometrium (particularly into the phospholipids) at the end of the oestrous cycle (Ning *et al.*, 1983). No significant differences in the amounts of any phospholipid in the guinea-pig endometrium between days 7 and 15 of



the cycle were found, indicating that the increased incorporation of arachidonic acid into endometrial phospholipids on day 15 compared to day 7 was not due to a change in phospholipid content (Ning and Poyser, 1984). The fact that there are no increases in either the *de novo* synthesis of PC, the synthesis of PC by methylation of PE or in the synthesis of PI, indicates that the increased incorporation of arachidonic acid into phospholipids between days 7 and 15 of the cycle cannot be due to an increase in phospholipid synthesis. The lack of changes in both PI content and PI synthesis between days 7 and 15 of the cycle, indicate that the stimulation of the PI cycle is not involved in the biochemical mechanisms that lead to increased  $\text{PGF}_{2\alpha}$  synthesis by the guinea-pig endometrium (Ning and Poyser, 1984). Therefore, the increased incorporation of arachidonic acid on day 15 probably occurs by acylation of lysophospholipids by the enzyme acyl-CoA:lysophospholipid acyltransferase (ACLS), as previously proposed (Ning *et al.*, 1983). This is in agreement with the findings of Trotter and Ferber (1981) who proposed that "the rapid incorporation of fatty acids into membrane phospholipids appears to be due to a turnover of fatty acyl chains rather than the *de novo* synthesis of phospholipids". The findings of other studies suggest that arachidonic acid is incorporated exclusively by this mechanism (Hill and Lands, 1968; Yamashita *et al.*, 1973). The increase in arachidonic acid uptake into endometrial phospholipids observed on day 15 of the guinea-pig oestrous cycle could therefore be due to an increase in ACLS activity, and/or changes in the amounts of lysophospholipid present and/or an increase in the activity of acyl-CoA synthetase (ACS). In comparison to arachidonic acid, the uptake of oleic acid into phospholipids was found to be unchanged between days 7 and 15, indicating a specific uptake for arachidonic acid. The ACLS present in rat liver has been shown to be relatively specific for arachidonyl-CoA; it is eight times more active with arachidonyl-CoA than with oleoyl-CoA as substrate (Yamashita *et al.*, 1973). Also, a study by Okuyama *et al.* (1975) demonstrated that different enzymes or different sites on a single enzyme exist for the transfer of different acyl-CoAs to lysophospholipids in rat liver. Platelets have also been shown to contain an ACS specific for arachidonic acid (Wilson *et al.*, 1982). Thus, if a specific increase in the enzymes ACLS and ACS specific for arachidonyl-CoA and arachidonic acid, respectively were to occur toward the end of

the oestrous cycle in the guinea-pig endometrium, this could explain the specific increase in the incorporation of arachidonic acid into endometrial phospholipids observed on day 15 of the cycle.

Although an increase in the rate of arachidonic acid uptake (i.e. reacylation into phospholipids) may be beneficial in providing a faster turnover of arachidonic acid for PG synthesis, such an increase may actually limit PG synthesis. Following its release by PLA<sub>2</sub>, the amount of free arachidonic acid would be reacylated at a faster rate if the uptake processes for arachidonic acid were stimulated. Indeed, this could be of benefit during early pregnancy, when PG output, particularly of PGF<sub>2α</sub>, from the uterus must be kept low so that luteal function is maintained. Studies on macrophages and lung fibroblasts demonstrated that inhibition of ACS and ACLS activities increased the concentration of free arachidonic acid and the amounts of PGs synthesized (Kroener *et al.*, 1981; Hunter *et al.*, 1984; Goppelt-Streube, 1986). In a more recent study (Preuss and Patscheke, 1992), the activities of PLA<sub>2</sub>, ACS and ACLS (lysophosphatidyl acyltransferase) were shown to be simultaneously active, resulting in a steady turnover of arachidonic acid in homogenates of human platelets. EGTA (a calcium chelator) suppressed the deacylating activity of PLA<sub>2</sub> on endogenous membrane phospholipids and prevented the formation of eicosanoids from previously esterified exogenous arachidonyl-CoA. Inhibition of reacylation of arachidonic acid by apyrase resulted in an increase in eicosanoid release (Preuss and Patscheke, 1992). These studies support the concept that eicosanoid release is not only controlled by an activation of PLA<sub>2</sub> but also by an inhibition of the reacylating enzymes ACS and ACLS in stimulated tissues.

Experiment 3:1 demonstrated that ACS, ACLS and PLA<sub>2</sub> activities were present in the guinea-pig endometrium on days 7 and 15 of the oestrous cycle. It may be possible that these ACS and ACLS activities have a role in the control of arachidonic acid turnover in the guinea-pig endometrium. If endometrial ACS and ACLS activities were to increase between days 7 and 15 of the cycle this could explain the previously observed increase in arachidonic acid uptake (into phospholipids) on day 15 of the cycle (Ning *et al.*, 1983). ACS and ACLS activities may be present due to a specific stimulation of their activities, or occur as a

consequence of the large quantity of free arachidonic acid being released by PLA<sub>2</sub> which requires reacylation, since free arachidonic acid is usually maintained at low concentrations (Bills, Smith and Silver, 1977).

Treatment with a PLA<sub>2</sub> inhibitor (i.e. ARA) of day 7 and day 15 non-pregnant guinea-pig endometrium cultured for 24 h (experiment 3:4) reduced the outputs of PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>, providing further evidence for the crucial role PLA<sub>2</sub> plays in the control of PG, particularly PGF<sub>2α</sub>, synthesis in the guinea-pig endometrium towards the end of the oestrous cycle. The decrease in the output of 6-keto-PGF<sub>1α</sub> was much less marked than the decrease in the output of PGF<sub>2α</sub> following PLA<sub>2</sub> inhibition. These findings would suggest that the syntheses of PGF<sub>2α</sub> and PGI<sub>2</sub> (as detected by 6-keto-PGF<sub>1α</sub>) are controlled differently. These findings are in agreement with previous studies (Riley and Poyser, 1987; 1990; Poyser, 1991).

The increase in 6-keto-PGF<sub>1α</sub> output from guinea-pig endometrium following treatment with HMB and THM during 24 h of culture indicates that the control of endometrial 6-keto-PGF<sub>1α</sub> synthesis in the non-pregnant guinea-pig is under the influence of arachidonic acid reacylation (results from 3:4). This is because, following inhibition of ACS and ACLS, the rate of arachidonic acid uptake into lysophospholipids will be reduced, thus increasing the amount of free arachidonic acid available for PG synthesis. In contrast, inhibition of ACS and ACLS did not increase, but actually decreased the outputs of PGF<sub>2α</sub> and PGE<sub>2</sub>. This would imply that the synthesis of these two PGs is not influenced by the activities of ACS and ACLS in the same way as that of the synthesis of 6-keto-PGF<sub>1α</sub>. However, during long term inhibition of ACS and ACLS, since the rate of arachidonic acid uptake into lysophospholipids will be reduced, the amount of arachidonic acid appropriately placed in the *sn*-2 position of appropriate phospholipids (PC and PE) for PLA<sub>2</sub> will also be eventually reduced. Therefore, PLA<sub>2</sub> may be indirectly inhibited by a lack of appropriate substrate. Since the syntheses of PGF<sub>2α</sub> and PGE<sub>2</sub> and that of 6-keto-PGF<sub>1α</sub> are different, this would explain the difference in the results of this study.

Experiment 3:2 demonstrated that ACS, ACLS and PLA<sub>2</sub> activities were also present in the endometrium of ovariectomized and ovarian steroid-treated

ovariectomized guinea-pigs. It would be of interest to know whether oestradiol acting on a progesterone-primed uterus (the physiological stimulus for increased secretion of uterine  $\text{PGF}_{2\alpha}$  towards the end of the oestrous cycle) could influence the activities of ACS, ACLS and  $\text{PLA}_2$ . If a decrease in ACS and ACLS activities were to occur towards the end of the cycle, this could be a possible mechanism for maintaining elevated concentrations of free arachidonic acid (since arachidonic acid would be reacylated at a slower rate) available for increased  $\text{PGF}_{2\alpha}$  synthesis at this time. Since oestradiol acting on a progesterone-primed uterus is believed to be the stimulus for increasing intracellular  $\text{Ca}^{2+}$  concentrations in guinea-pig endometrial cells which is then responsible for activating  $\text{PLA}_2$  (Downing and Poyser, 1983), it would be interesting to study whether oestradiol treatment of progesterone-primed ovariectomized guinea-pigs results in an increase in endometrial  $\text{PLA}_2$  activity.  $\text{PLA}_2$  activity in the guinea-pig endometrium increases 1.8-fold between days 7 and 16 of the oestrous cycle (Downing and Poyser, 1983). It is not clear whether such an increase occurs in the sheep, however, there is no change in endometrial  $\text{PLA}_2$  activity in the ewe between days 12, 14 and 16 of the cycle (Tamby, Charpigny, Reinaud and Martal, 1993). The administration of oestradiol to hypophysectomized rats resulted in a large stimulation of uterine  $\text{PLA}_2$  activity. Progesterone pre-treatment substantially reduced this stimulatory effect of oestradiol, but did not abolish it (Dey, Hoversland and Johnson, 1982). Thus, oestradiol acting on a progesterone-primed uterus may be the stimulus for increased endometrial  $\text{PLA}_2$  activity in the guinea-pig and other species.

A  $\text{PLA}_2$ -stimulating protein (PLAP) which is antigenically and functionally related to the  $\text{PLA}_2$ -stimulating protein melittin, has been isolated from a variety of cell lines and is an intracellular messenger in the stimulation of PG synthesis (Clark *et al.*, 1987; Clark *et al.*, 1988). Indeed, melittin does stimulate  $\text{PGF}_{2\alpha}$  output from the guinea-pig uterus, suggesting that a protein that stimulates  $\text{PLA}_2$  could be present in guinea-pig endometrium (Johnson and Poyser, 1991). PLAP has a molecular weight of 28 kDa and is detectable in human rheumatoid arthritis synovial fluid (Bomalaski *et al.*, 1990). It is unclear what the stimulus for PLAP is and how exactly PLAP acts to activate  $\text{PLA}_2$ . It has been suggested that PLAP may affect the genome and thus



regulate the formation of both lipocortins and phospholipases (Dennis, 1983). The guinea-pig endometrium does not secrete a protein of 28 kDa size during 24 h culture (Leckie and Poyser, 1993), however, even if PLAP were produced by the guinea-pig endometrium *in vivo*, it may not be a secreted protein and would thus be undetected under such experimental conditions. Thus, at the present time, it is unknown whether PLAP is present in the guinea-pig endometrium, and if it were, whether it could be involved in the stimulation of endometrial  $\text{PGF}_{2\alpha}$  synthesis towards the end of the oestrous cycle.

In the pregnant guinea-pig, the increase in uterine  $\text{PGF}_{2\alpha}$  production which normally occurs after day 11 of the cycle, must be prevented since continued progesterone is necessary for the first one third of gestation in the guinea-pig (Bland and Donovan, 1969b). The presence of the conceptuses has been shown to prevent uterine  $\text{PGF}_{2\alpha}$  output (Blatchley *et al.*, 1975b). The conceptuses apparently secrete an anti-luteolytic factor which acts to suppress uterine  $\text{PGF}_{2\alpha}$  output by preventing the stimulatory action of oestradiol on endometrial  $\text{PGF}_{2\alpha}$  synthesis and release (Poyser, 1984a). This anti-luteolytic factor acts predominantly in a local manner, since in unilaterally pregnant guinea-pigs with the sterile horn transected at the cervical end, uterine  $\text{PGF}_{2\alpha}$  synthesizing capacity is 3-fold higher and the corpora lutea half the size on the sterile side compared to the pregnant side (Maule Walker and Poyser, 1974). The identity of the anti-luteolytic factor in the guinea-pig is not known, but it is probably different from oTP-1 and bTP-1 which are proteins produced by ovine and bovine embryos, respectively, that inhibit endometrial  $\text{PGF}_{2\alpha}$  synthesis (Bazer, Vallet, Roberts and Sharp, 1986). These trophoblast proteins are structurally related and have similar activity to  $\alpha$ -interferon (Imakawa *et al.*, 1987; Stewart *et al.*, 1987; Charpigny *et al.*, 1988) but are now classed separately as interferon- $\tau$ . However, the anti-luteolytic factor secreted by the guinea-pig conceptus is not an interferon and is most probably different from oTP-1 (Leckie and Poyser, 1990a). Interestingly, those species whose embryos secrete a trophoblast protein-1 are the same species in which ovarian oxytocin forms part of the physiological stimulus for the increase in endometrial  $\text{PGF}_{2\alpha}$  synthesis towards the end of the oestrous cycle.

It is not known whether the presence of the conceptuses in the guinea-pig alters the control of arachidonic acid turnover during pregnancy. An increase in the rate of arachidonic acid uptake may limit the amount of free arachidonic acid available for PG synthesis and therefore reduce uterine PG output. Experiment 3:3 demonstrated that ACS, ACLS and PLA<sub>2</sub> activities were also present in the endometrial and conceptual tissues of day 15, 29 and 36 pregnant guinea-pigs. Whether an increase or decrease in the activities of ACS, ACLS and PLA<sub>2</sub> in endometrial or conceptual tissues may be a mechanism by which PG production is controlled during pregnancy is unknown. Experiment 3:5 was carried out in order to investigate the effects of inhibitors of the enzymes that control arachidonic acid turnover and the actual quantities of PGs that are produced by the endometrial and conceptual tissues during pregnancy in the guinea-pig.

Similarly to the results from experiment 3:4 in which non-pregnant endometrium was used, inhibitors of ACS and ACLS decreased PGF<sub>2α</sub> and PGE<sub>2</sub> outputs, and increased the output of 6-keto-PGF<sub>1α</sub> from the endometrium of day 22, 29 and 36 pregnant guinea-pigs maintained in culture for 24 h. The inhibitor of PLA<sub>2</sub> also reduced all three endometrial PG outputs on all days of pregnancy. Therefore, the control of arachidonic acid turnover appears to be similar in the non-pregnant and pregnant guinea-pig endometrium.

In contrast to the endometrium, in the subplacenta, inhibitors of arachidonic acid reacylation increased PGF<sub>2α</sub> output and, to a lesser extent, PGE<sub>2</sub> output. The output of 6-keto-PGF<sub>1α</sub> was unaffected in early-pregnant (day 22) animals, but slightly increased on day 36 of pregnancy. ACS and ACLS appear to be involved in the control of PG synthesis from this tissue. The inhibitor of PLA<sub>2</sub> had no effect on PGF<sub>2α</sub> output (apart from a slight decrease in day 29 pregnant animals) from the subplacenta. Therefore, PLA<sub>2</sub> does not appear to be involved in the release of free arachidonic acid for PGF<sub>2α</sub> synthesis in the pregnant guinea-pig subplacenta. Treatment with a PLA<sub>2</sub> inhibitor actually resulted in an unexpected increase in the outputs of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>. The reason for this is not clear, but could may be due to other effects of the inhibitor on PG synthesis and metabolism.

In the chorio-allantoic placenta from pregnant guinea-pigs maintained in culture for 24 h, the inhibitors of arachidonic acid uptake slightly increased  $\text{PGF}_{2\alpha}$  output, but only in day 29 and 36 pregnant animals and only after 24 h of culture. The output of  $\text{PGE}_2$  and 6-keto- $\text{PGF}_{1\alpha}$  were also increased, but only after 18 and 24 h of culture and on days 22 and 29, not day 36, of pregnancy. Therefore, ACS and ACLS do appear to be involved in the control of PG synthesis by the chorio-allantoic placenta, but not to such an extent as that observed in the subplacenta. The inhibitor of  $\text{PLA}_2$  in the chorio-allantoic placenta inhibited  $\text{PGF}_{2\alpha}$  and  $\text{PGE}_2$  outputs slightly on day 22 of pregnancy, but had very little effect on PG output in later pregnant animals. Similarly to the subplacenta, inhibition of  $\text{PLA}_2$  in day 29 and 36 pregnant guinea-pigs actually tended to increase PG output slightly. Therefore, in the guinea-pig subplacenta and chorio-allantoic placenta, the control of arachidonic acid uptake does appear to be involved in the control of PG output from these tissues during pregnancy. In contrast,  $\text{PLA}_2$  does not appear to have a role in the regulation of arachidonic acid for PG synthesis in these tissues.

In the fetal membranes, inhibition of ACS and ACLS increased  $\text{PGF}_{2\alpha}$  (both day 29 and 36 pregnant animals) and  $\text{PGE}_2$  (only day 29 pregnant animals) outputs, but only after 24 h of culture. The output of 6-keto- $\text{PGF}_{1\alpha}$  was slightly decreased on day 29, but greatly increased by THM treatment on day 36 of pregnancy, suggesting a role for the control of arachidonic acid uptake in the regulation of PG, particularly 6-keto- $\text{PGF}_{1\alpha}$ , output from the fetal membranes of pregnant guinea-pigs. The inhibition of  $\text{PLA}_2$  tended to inhibit all PG outputs from the fetal membranes.

In section 3:5, the basal PG outputs from the endometrium, subplacenta, chorio-allantoic placenta and fetal membranes of day 22, 29 and 36 pregnant guinea-pigs during 24 h culture were also measured. These results indicated that  $\text{PGF}_{2\alpha}$  and 6-keto- $\text{PGF}_{1\alpha}$  outputs were generally greatest from the endometrium, with much lesser amounts produced by the subplacenta, chorio-allantoic placenta and fetal membranes.  $\text{PGE}_2$  output was similar from all tissues. However, there was a striking 14-fold increase in  $\text{PGF}_{2\alpha}$  output from the chorio-allantoic placenta between days 22 and 36 of pregnancy. Therefore, the chorio-allantoic placenta may be an important source of  $\text{PGF}_{2\alpha}$  later in pregnancy.



It may be possible that the low amounts of PGs synthesized by the fetal membranes are due to a stimulation of the mechanisms that control arachidonic acid uptake. Such a stimulation may limit the amount of free arachidonic acid available for PG synthesis, resulting in the low outputs observed in section 3:5. In contrast, in the chorio-allantoic placenta, where greater amounts of all PGs, particularly  $\text{PGF}_{2\alpha}$ , are produced later in pregnancy, low ACS and ACLS activities could reduce the rate of arachidonic acid uptake thus increasing the amount of free arachidonic acid available for PG synthesis.

The control of arachidonic acid turnover may play an important role in the regulation of PG synthesis in the guinea-pig uterus. Perhaps oestradiol acting on a progesterone-primed uterus, the physiological stimulus for increased  $\text{PGF}_{2\alpha}$  output from the endometrium towards the end of the cycle for the purpose of luteal regression, could inhibit the rate of arachidonic acid reacylation on day 15 of the cycle which could, in turn, be of benefit in maintaining elevated concentrations of free arachidonic acid available for increased PG synthesis at that time. Further studies are needed to investigate these possibilities.

PGs have an important role during pregnancy. They subserve the establishment, maintenance and conclusion of pregnancy (Boone, Currie and Leung, 1993). In the human, successful implantation and maintenance of pregnancy involves trophoblast invasion of the decidualized endometrial stroma and dilatation of maternal spiral arteries that terminate in intervillous spaces. These events depend on increased blood flow to the feto-placental unit, inhibition of clotting at the maternal-placental interface and suppression of the maternal immune response (North, Whitehead and Larkins, 1991).

Maintenance of a PG balance may be critical to fetal and placental blood perfusion. Placental insufficiency could lead to hypoxic / ischemic conditions damaging fetal development.  $\text{PGI}_2$  from fetal vessels of the placenta has been shown to be involved in the maintenance of the low pressure, high velocity state of the human fetal placental circulation. The fetal vasculature preferentially produces  $\text{PGI}_2$  rather than any other PG, and at higher concentrations than maternal vessels (Gude, Rice, King, Boura and Brennecke, 1990). Arachidonic acid or  $\text{PGI}_2$  infusion results in

vasodilation of fetal vessels of human perfused placental lobules while arachidonic acid results in vasoconstriction of umbilical veins (Gude *et al.*, 1990). PGI<sub>2</sub> is a major product of cultured human umbilical vein endothelial cells (Patel, Sullivan and Elder, 1989).

PGs originating from the conceptus appear to have numerous roles, including, intrauterine migration of embryos, blastocyst hatching from the zona pellucida, ion transport across trophoctoderm, fluid accumulation in the blastocoele, increased endometrial capillary permeability and blastocystic glucose metabolism (see Lewis, 1989). Between days 7 and 12 of pregnancy, pig embryos migrate and become spaced in the uterus. Local myometrial contractions appear to be involved in controlling this migration, and blastocystic PGs may stimulate these contractions (Pope, Maurer and Stormshak, 1982). Before blastocyst implantation, its protective covering, the "zona pellucida" must be shed. It has been demonstrated that inhibitors of PG synthesis and PG antagonists reduce the rate of hatching of mouse embryos from the zona pellucida (Biggers, Leonov, Baskar and Fried, 1978). Since PGE<sub>2</sub> is able to promote water movement across epithelia, it was proposed that PGE<sub>2</sub> produced by the blastocyst promotes water passage across the trophoctoderm resulting in the accumulation of blastocoelec fluid, and the eventual "hatching" of the blastocyst from the zona pellucida (Biggers *et al.*, 1978). Later studies supported this hypothesis (Hurst and Farlane, 1981; Chida, Uehara, Hoshiai and Yajima, 1986). In a study by Lewis (1986b) it was demonstrated that indomethacin decreased PG synthesis by, and transport of <sup>22</sup>Na across, day 16 sheep trophoblast and also prevented the formation of trophoblastic vesicles *in vitro*. This study suggested that PGs may be involved in the control of fluid accumulation during the period of rapid blastocystic elongation (prior to implantation) in sheep. Localized increases in endometrial capillary permeability precede implantation in numerous mammals, and PGs appear to be involved in initiating these increases (see Lewis, 1989). Other studies have also suggested a role for PGs (possibly of blastocystic origin) in the control of energy metabolism in blastocysts (see Lewis, 1989).

During pregnancy, a balance must exist between immune tolerance of fetal tissue and a local suppression of inflammatory responses. It has been suggested that

progesterone, with its immunosuppressive and anti-inflammatory properties, plays a role in maintaining this balance. Evidence for this theory is provided by studies using antiprogesterone steroids, such as RU486, that can disrupt pregnancy in women at any stage and, used together with PGs, provide an effective method of inducing abortion (Kelly, 1994). A possible mechanism of controlling PG concentrations may be through prostaglandin dehydrogenase (PGDH), the enzyme responsible for the conversion of PGs to their 15-keto metabolites, and thus inactivating them. This enzyme is abundant in the pregnant uterus, and could provide a mechanism of control in maintaining low concentrations of PGs during pregnancy, and increased PG concentrations during the process of parturition. Indeed, PGDH rises in early pregnancy, and some of the highest concentrations of this enzyme are seen in chorion, decidua and placenta (Kelly, 1994). In women at term elective cesarean section or after term spontaneous labor immunoreactive PGDH was demonstrated to be predominant in chorion, present at low activities in decidua and absent in amnion. PGDH in the placenta was localized in intermediate trophoblast cells and syncytiotrophoblast, but not in cytotrophoblast or villous stromal tissue. (Cheung, Walton, Tai, Riley and Challis, 1990). There was no change in the localization of PGDH in any of the tissues in association with labor, suggesting that changes in PGDH activity or distribution are unlikely to contribute to the increase in PG concentration at parturition. However, it may be possible that placental PGDH could influence the concentration of PGs produced within the placenta which reach the underlying decidua and/or myometrium. During late human pregnancy, the amnion is believed to be a major site of PGE<sub>2</sub> production, which may cross the fetal membranes to exert biological effects on decidua and/or myometrium. A high PGDH activity in the chorion would suggest a possible role in the maintenance of pregnancy, protecting the decidua from fetal-derived PGs. However, PGDH is present heterogeneously in chorion and thus may not present the uniform PG metabolic barrier previously assumed, since some PG may cross and escape metabolism (Cheung *et al.*, 1990). Other studies have demonstrated PG production by sheep trophoblast (Ellwood, Mitchell, Anderson and Turnbull, 1979) and studies in placental tissue at term showed PGDH at high concentrations in intermediate trophoblast and syncytiotrophoblast, but

syncytiotrophoblast, but not in cytotrophoblast (Challis, Jacobs, Riley, Boshier, Han, Smith, Cheung, Langlois and Fraher, 1990; Cheung, Walton, Tai, Riley and Challis, 1990). However, in first trimester human villous trophoblast, the PG synthetic capacity of the syncytiotrophoblast is associated with extensive PGDH localized in the cytotrophoblast (Cheng *et al.*, 1993a). This combination results in the expression of PGE in the syncytiotrophoblast (Cheng *et al.*, 1993b) with a presumed outward secretion and would provide a mechanism by which vessels of the fetal circulation lying within the trophoblastic villi are protected against PGs (Kelly, 1994). A high concentration of PGE in the syncytiotrophoblast layer could act as an immunosuppressive defence against maternal blood cells (Tawfik, Hunt and Wood, 1986). The trophoblastic villi show only weak staining for progesterone receptors (Shi, Wang, Fu, Xu and Zhu, 1993) and thus this source of PGE is unlikely to be under the influence of progesterone.

PGDH levels dropped markedly in guinea-pigs treated with RU486, especially in tissue with a high metabolizing capacity such as chorio/decidual tissue (Kelly and Bukman, 1990). Enzyme measurements in tissue from women given RU486 showed a similar drop in PGDH activity, with significant decreases following 12 h exposure to RU486. (Cheng, Kelly, Thong, Hume and Baird, 1993a). Immunohistochemistry studies demonstrated that the drop in PGDH was seen in all cells, but particularly in those that were perivascular (Cheng, Kelly, Thong, Hume and Baird, 1993b). When PGE was examined in decidua from these women, the elevation in PGE in small blood vessels was striking. This increase was significant following 12 h RU486 treatment *in vivo*, and was accompanied by a decrease in observable PGE metabolite. Small blood vessels that were PGE negative and 13,14-dihydro-15-keto-PGE<sub>2</sub> (PGEM) positive in untreated women, were PGE positive and 13,14-dihydro-15-keto-PGE<sub>2</sub> (PGEM) negative in RU486-treated women. These results would suggest that PG stimulation by the antiprogestin RU486, is by means of a direct effect on PGDH, and that the prominent increase in PGE in blood vessels may be a major mode of action in causing abortion, and that one of the major mechanisms by which progesterone ensures a successful pregnancy is by maintenance of low PG levels in the vasculature. The PGs may also synergize with leukocyte chemotactic agents stimulating neutrophil ingress

and tissue destruction (Cheng *et al.*, 1993). A significant signal for immigration of neutrophils into tissues, from a chemokine such as interleukin (IL)-8, which also has activating potential itself, will stimulate the release of their specific granules, containing collagenase, which can dissolve and disrupt extracellular matrix and ground substance. Further activation can result in the release of proteases, leukotrienes, PGs and a wide range of effector cytokines, such as IL-8 (see Kelly, 1994). This may be of importance during the process of parturition.

The conceptus must be able to modulate the maternal humoral (antibody-mediated) and cellular (killer-cell mediated) immune responses. Several mechanisms that have been demonstrated appear to utilize PGE<sub>2</sub>. An immunosuppressive agent produced by cultured human decidual cells has been identified as PGE (Parhar, Kennedy and Lala, 1988). PGE<sub>2</sub> inhibits the function of many cells of the immune system, and in human semen (with the highest concentrations of PGE found so far), PGE is believed to suppress the immune responses of the female reproductive tract to maximize the chances of reproductive success (Kelly, 1994). The mechanism of action of PGE on leukocytes involves an increase in intracellular cAMP concentrations. The major PGs in human semen, the 19-hydroxy PGEs, are selective EP2 receptor agonists which are associated with a rise in intracellular cAMP, thus providing further evidence for the immunosuppressive role of the seminal PGs (Kelly, 1994). Although the majority of effects of PGE on leukocytes are suppressive, there is new evidence that PGE may act synergistically with IL-2 to stimulate granulocyte macrophage colony stimulating factor (GM-CSF) production (Quill, Gaur and Phipps, 1989), which has also been demonstrated to stimulate placental cell growth (Athanasakis, Bleackley, Paetkan, Guilbert, Barr and Wegmann, 1987; Armstrong and Chaouat, 1989).

A feature of first trimester decidua is the increased quantity of large granular lymphocytes (LGL) attributed to the Natural Killer (NK) cell lineage that display an abundance of a characteristic marker (CD56) (Ritson and Bulmer, 1987). These cells differ from the majority of circulating NK cells in their morphology, cell surface markers and cytotoxicity. LGLs in decidua have been demonstrated to have low cytotoxicity and not to kill trophoblast cells (King and Loke, 1990), but this situation



can be reversed by IL-2 treatment. The specific mechanism responsible for maintaining or inducing the phenotype and morphology of these regulatory cells is unclear, but recent experiments have implicated a role for PGE<sub>2</sub>. Cytotoxicity in NK cells can be reduced by PGE<sub>2</sub> (Goto, Herberman and Holden, 1983). The cells found in murine decidua basalis in close proximity to the trophoblast are, similarly to their human counterparts, of the NK lineage (Linnemeyer and Pollack, 1991a); large and granular with poor cytotoxicity, but killing can be induced by IL-2. Mouse LGLs exhibit a particular marker (4H12) of large granular phenotype (Linnemeyer and Pollack, 1991b). In a study where murine spleen-derived adherent NK cells were treated with PGE in culture, the cells underwent transition to terminally differentiated NK cells exhibiting the large granular morphology and 4H12 marker. The cells gaining the 4H12 marker demonstrated a greatly reduced cytotoxicity (Linnemeyer and Pollack, 1993). Thus, PGE<sub>2</sub> appears to play a role in the transformation of the function, morphology and markers of NK cells in the mouse. Evidence for such a role of PGs is further strengthened by a study in which chronic indomethacin treatment to pregnant mice resulted in NK cell activation within the uterus and subsequent embryo death (Lala, Scodras, Graham, Lysiak and Parhar, 1990). It is presently unknown whether a similar mechanism may occur in the human and other species. Another action of PGE in reducing the effect of the maternal immune response is its ability to induce "class switching" of globulins, so that the production of immunoglobulin (Ig) G-1 and IgE are favored with a subsequent reduction in the ability to activate the complement system (Phipps, Stein and Roper, 1991). Also, paternal antigens are produced in several species, including man, and can be induced in mice. In mice, paternal antigens are almost exclusively of the IgG-1 isotype, which because it is unable to fix complement, is not very cytotoxic, with no cytotoxicity having been detected in maternal sera (Bell and Billington, 1980). A recent hypothesis based on studies of decidual cytokine ratios in mice, suggests that pregnancy is characterized by a strengthened maternal humoral immune response and a diminished cellular response. This is characterized by an increase in T helper (Th) -2 cells (which produce cytokines that stimulate antibody generation) and a decrease in Th-1 cell activity (which generate cytokines required for cellular responses) (Wegmann, Lin, Guilbert and

Mosmann, 1993). In an inflammatory response, Th-1/Th-2 responses are determined by antibody presenting cells, and the mediator is believed to be PGE<sub>2</sub>. PGE stimulates cAMP in both Th-1 and Th-2 cells, however, such a change only results in the inhibition of Th-1 cells (Betz and Fox, 1991). Therefore, a physiological control mechanism that is observed in inflammation, may be used within the decidua, where PGE could be produced by either decidual cells or syncytiotrophoblast.

The present studies have demonstrated that the control of arachidonic acid turnover may have a role in the regulation of PG synthesis during pregnancy in the guinea-pig. In particular, the control of arachidonic acid uptake may be important in the placentae and fetal membranes, but not in the endometrium, of pregnant guinea-pigs. Also, PLA<sub>2</sub> may have a role in the endometrium and fetal membranes, but not in the placentae, in the regulation of PG synthesis from the pregnant guinea-pig.

In a study by Riley and Poyser (1987), it was proposed that in the non-pregnant guinea-pig, oestradiol induces the synthesis of a protein ("lipostimulin") by the endometrium which acts on a progesterone-primed uterus to raise the endometrial intracellular Ca<sup>2+</sup> concentration. This calcium then associates with calmodulin (Poyser, 1985a, b) which then activates PLA<sub>2</sub> (Moskowitz *et al.*, 1985). Thus, PLA<sub>2</sub> will release arachidonic acid from endometrial phospholipids, mainly PC and PE (Leaver and Poyser, 1981; Ning and Poyser, 1984) resulting in increased PG synthesis. The calcium ionophore, A23187 stimulates uterine PG synthesis by a mechanism dependent upon the presence of calcium (Poyser, 1984b). The effect of A23187 on arachidonic acid turnover inhibited- PG synthesis from the day 7 superfused guinea-pig uterus *in vitro*, was investigated in experiment 3:6. Inhibitors of ACS, ACLS and, surprisingly, PLA<sub>2</sub> all increased PGF<sub>2α</sub>, PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> outputs. The inhibition of ACS and ACLS presumably slowed the rate of arachidonic acid uptake thus, increasing free arachidonic acid available for PG synthesis. The reason for the increase in PG output following PLA<sub>2</sub> inhibition is unclear, but may be due to other effects of the inhibitor on PG synthesis and metabolism. The increase in PG synthesis following PLA<sub>2</sub> inhibition and A23187 treatment may be due to A23187 stimulating PLC activity.



Inhibition of ACS and ACLS with THM prevented the A23187-stimulated increase in PG output. The reason for this is unclear. The inhibition of arachidonic acid uptake may lead to a reduction in an appropriate phospholipid pool for the action of PLA<sub>2</sub>, since arachidonic acid is not taken up into lysophospholipids if ACS and ACLS are inhibited. This would explain the increase in PG output during the first 20 min of THM treatment, and the lack of effect of A23187 during the following 20 min when the uterus was still treated with THM. This would also explain why inhibitors of arachidonic acid turnover decreased endometrial PGF<sub>2α</sub> and PGE<sub>2</sub> outputs during 24 h of culture i.e. over a long period of inhibition of arachidonic acid uptake, appropriate phospholipids (PC and PE containing *sn*-2 arachidonic acid) will become limited, thus reducing PG synthesis. The syntheses of PGF<sub>2α</sub> and PGE<sub>2</sub> and the synthesis of 6-keto-PGF<sub>1α</sub> are different, explaining why the output of endometrial 6-keto-PGF<sub>1α</sub> is increased by the inhibition of ACS and ACLS during 24 h culture. It has been previously suggested that a different pool of arachidonic acid is used in the production of 6-keto-PGF<sub>1α</sub> by the guinea-pig endometrium (Poyser, 1991).

In experiment 3:7, arachidonic acid was not limiting during the 1 h incubation of day 7 guinea-pig homogenates together with arachidonic acid turnover inhibitors, since exogenous arachidonic acid was added to the Krebs solution in which the tissue was homogenised and incubated. Therefore, since free arachidonic acid was not limiting, it was expected that treatment with THM and HMB would have no effect on PG release by guinea-pig uterine homogenates. Perhaps the decrease in PGF<sub>2α</sub> output following THM treatment was due to inhibition of arachidonic acid uptake indirectly decreasing the appropriate phospholipid pool available for the action of PLA<sub>2</sub> i.e. in the guinea-pig uterus, the synthesis of PGF<sub>2α</sub> appears to depend upon the availability of phospholipids, predominately PC and PE, containing arachidonic acid at the *sn*-2 position. This does not appear to be the case for 6-keto-PGF<sub>1α</sub>, and to a lesser extent PGE<sub>2</sub>, output from the guinea-pig uterus since these PG outputs were unaffected by THM treatment of guinea-pig uterine homogenates. HMB was found to be much less potent than THM in other experiments, so that during this experiment, arachidonic acid probably continued to be reacylated, thus resulting in reduced PG synthesis. The increase in the output of all three PGs following treatment with a PLA<sub>2</sub> inhibitor

(ARA) is somewhat surprising. Perhaps the inhibition of PLA<sub>2</sub> “switches on” PLC activity since the guinea-pig uterus has a large source of a PLC enzyme specific for PI (Bennett and Crooke, 1987). Also, PGHS exhibits self-catalysed breakdown during the synthesis of PGs (Lands, 1979). Consequently, the total amount of PGs synthesized by endometrial homogenates is indicative of the concentration of PGHS, especially since the metabolism of PGs in the absence of NAD<sup>+</sup> by the guinea-pig uterus is negligible (< 5%) (Poyser, 1979). Thus, ARA treatment may have induced an increase in PGHS activity resulting in increased PG production in the homogenate experiments.

The four principle techniques used in these investigations, namely superfusion, culture, homogenate and microsome, have produced results with the aim of further understanding the control of arachidonic acid turnover in relation to PG production in the guinea-pig uterus. Results between different techniques cannot be directly related since all have different experimental conditions and used uterine tissues in different preparations. This is highlighted in the findings that short-term inhibition (as in superfusion experiments) of arachidonic acid reacylation resulted in generally increased PG production, possibly due to an increase in free arachidonic acid available for PG synthesis. However, during long term inhibition of arachidonic acid reacylation (as in 24 h tissue culture experiments) a general decrease in PG production was observed suggesting a decrease in PG synthesis. This may have been due to the indirect inhibition of PLA<sub>2</sub>, since during long term inhibition of arachidonic acid reacylation, appropriate esterified arachidonic acid in phospholipids will also be eventually reduced.

All the techniques used were not only carried out on different time scales (superfusion: 160 min, culture: 24 h, homogenate: 1 h, microsome: 10 min) but also utilized different tissue preparations. Superfusion utilized whole uterine tissue, so that the presence of the myometrium should not be disregarded. The short duration of drug treatments (40 min), immediate collection of PGs, together with simultaneously run controls, achieved clear observations of PG production, and the influence of different treatments on PG production, by the superfused guinea-pig uterus *in vitro*. The tissue culture experiments utilized endometrial and fetal tissues cultured for 24 h,

with or without drug treatments, and were sampled for PG production every 6 h. The influence of inhibitors of arachidonic acid turnover as seen in the resulting PG production was compared to PG production in untreated tissue. The possibility of toxic effects due to the inhibitors on the tissues during such long term experiments, and particularly at high inhibitor concentrations, should be considered. Cell-viability tests at the end of such experiments should ideally be carried out. In the experiments carried out using 1 h endometrial homogenate incubations, tissue was homogenized. Since the tissue was homogenized, the PG production measured represents that from disrupted cellular tissue. This may have specific effects on PG synthesis, such as inducing the activity of PGHS or influencing the metabolism of PGs. PGHS exhibits self-catalysed breakdown during the synthesis of PGs (Lands, 1979). Consequently, the total amount of PGs synthesized by endometrial homogenates is indicative of the concentration of PGHS, especially since the metabolism of PGs in the absence of  $\text{NAD}^+$  by the guinea-pig uterus is negligible ( $< 5\%$ ) (Poyser, 1979). Thus, this technique is useful for investigating the influence of drug treatments, not only on the resulting PG production, but also as a possible method for observing changes in PGHS activity in the guinea-pig uterus. In the experiments investigating the presence of the enzyme activities that control arachidonic acid turnover in the guinea-pig uterus microsome preparations were utilized. This achieves a specific investigation of the enzymes in their cellular compartments. However, the position of the enzymes is of importance, and this may vary for different enzymes, making comparison of results for different enzymes difficult. For instance, one enzyme on the inside of the endoplasmic reticulum will have more difficulty in reacting with exogenous substrates, than another enzyme positioned on the exterior of the endoplasmic reticulum. Using phospholipid mixtures in the reactions (as in these experiments) also raises the possibility of micelle formation, and thus difficulty for the enzyme-substrate complexes to form.

Other methods that could have been used to detect the presence of enzyme include Western blotting and using probes for mRNA of the enzymes. For example, by using quantitative Western blotting, the protein (enzyme) concentrations could have been investigated at different times, during the oestrous cycle and pregnancy, illustrating whether any changes in the concentrations of the enzymes occurs during

these times. If an antibody were available, the cellular site of the enzymes could also be shown using immunohistochemistry techniques. If cDNA sequences to these enzymes were known and available, expression of mRNA during the oestrous cycle and pregnancy could be examined by Northern blotting, and this mRNA localised using in situ hybridisation techniques.

The term “electrophoresis” describes the migration of a charged particle under the influence of an electric field. Many biological molecules, including proteins, possess ionisable groups, and thus, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-). By applying a potential difference, different proteins can be separated due to their different electrophoretic mobilities. The protein mixture is applied to some form of support media, commonly polyacrylamide gels. Hence polyacrylamide gel electrophoresis (PAGE). Separated samples can be located by staining with an appropriate dye or by autoradiography if the sample is radiolabelled. SDS-PAGE (using the detergent SDS) is frequently used for studying proteins. However, this method is of no use to detect one protein (such as an enzyme) since the SDS denatures the enzyme. Therefore in this case, it is necessary to use non-denaturing conditions. In native or buffer gels, polyacrylamide gels are used without SDS so that the proteins are not denatured prior to loading. Since all the proteins carry their native charge at the pH of the gel, proteins separate according to their different electrophoretic mobilities and the sieving effects of the gel. The enzyme of interest can be identified by incubating the gel in an appropriate substrate solution such that a coloured product is produced at the site of the enzyme. An alternative method for enzyme detection is to include the substrate in an agarose gel that is poured over the acrylamide gel and allowed to set. Diffusion and interaction of enzyme and substrate between the two gels results in colour formation at the site of the enzyme (Wilson and Walker, 1994). Although essentially an analytical technique, PAGE does achieve fractionation of a protein mixture during the electrophoresis process. It is possible to make use of this fractionation to examine further individual separated proteins. The first step is to transfer or “blot” the pattern of separated proteins from the gel onto a sheet of nitrocellulose paper. This method is known as “Western blotting”, by analogy with “Southern blotting”, the equivalent method used



to recover DNA samples from an agarose gel. Transfer of the proteins from the gel to the nitrocellulose can be achieved in one of two ways. In “capillary blotting” the gel is placed on a wet pad of buffer-soaked filter paper and a sheet of nitrocellulose placed on the gel. Buffer is then drawn through the gel by placing a pad of dry absorbent material (such as filter paper) followed by a heavy weight on top of the nitrocellulose sheet. The passage of buffer by capillary action through the gel carries the separated proteins onto the nitrocellulose sheet, to which they bind irreversibly by hydrophobic interaction. The process is carried out overnight, but due to the small pore size of the acrylamide gel, only a limited amount of buffer travels through the gel in this time, so that only between 10-20 % of the protein in the gel is transferred. A quicker and more efficient method of transfer is achieved by “electroblotting” where a sandwich of gel and nitrocellulose is compressed in a cassette and immersed, in buffer, between two parallel electrodes. A current is passed at right angles to the gel, which causes the separated proteins to electrophorese out of the gel and onto the nitrocellulose sheet. The nitrocellulose with its transferred protein is referred to as the “blot”. Once transferred onto the nitrocellulose, the separated proteins can be examined further. This involves “probing” the blot, often using an antibody to detect a specific protein. The blot is firstly incubated in a protein solution (eg. BSA) which will block all remaining hydrophobic binding sites on the nitrocellulose sheet (referred to as “blotto technique”. The blot is then incubated in a dilution of an antiserum (primary antibody, eg. rabbit IgG) directed against the protein of interest. The blot is then incubated further with a second antibody directed against the IgG of the species that provided the primary antibody (eg. anti-rabbit IgG). This second antibody is appropriately labelled so that the interaction of the secondary antibody to the primary antibody can be visualised. A common detection method is to use an enzyme-linked second antibody, so that following incubation of the blot with second antibody, the blot is further incubated in enzyme-substrate solution. The enzyme converts the substrate into an insoluble coloured product that is precipitated onto the nitrocellulose. The presence of the coloured band indicates the position of the protein of interest. Although enzymes are commonly used as markers for second antibodies, other markers are also used including,  $^{125}\text{I}$ -labelled second antibody (binding to the blot is

detected by autoradiography), fluorescein isothiocyanate-labelled second antibody (fluorescent label detected by exposure of blot to ultra-violet light),  $^{125}\text{I}$ -labelled Protein A (Protein A binds to IgG, thus  $^{125}\text{I}$ -labelled Protein A is used instead of second antibody, and binding to blot is detected by autoradiography) and gold-labelled second antibody (directly visible as red colour when gold particles bind to primary antibody on the blot) (Wilson and Walker 1994; Burnette, 1981). Hybridization of a nucleic acid probe to endogenous mRNA present in cytological or histological preparations is one of the most precise methods available for detecting target mRNA (Decimo, Labouesse and Dolle, 1995). mRNA contains sequences of ribonucleotides which code for amino acid sequences of proteins. A single mRNA molecule codes for a single polypeptide chain in eukaryotes (Wilson and Walker, 1994). Thus, the *in situ* hybridization of a nucleic acid probe to a particular cellular mRNA allows the detection of the particular protein (eg. enzyme) coded by that mRNA. At present, complementary RNA (cRNA) probes (riboprobes) labelled with a low-energy  $\beta$  emitter are commonly used as they are believed to offer the greatest sensitivity (Decimo *et al.*, 1995).

In summary, these studies have investigated the role that the control of arachidonic acid turnover plays in the control of PG synthesis in the non-pregnant and pregnant guinea-pig uterus. The important roles of PGs, both in the non-pregnant and pregnant guinea-pig uterus, have been discussed. ACS, ACLS and  $\text{PLA}_2$  activities have been demonstrated to be present in both non-pregnant and pregnant endometrium, and in the conceptual tissues of pregnant guinea-pigs. Thus, these enzymes may play a part in the control of arachidonic acid turnover and therefore, PG synthesis, in these tissues. The use of inhibitors has also demonstrated that these enzymes may be involved in the tightly regulated control of PG production during the oestrous cycle and early pregnancy in the guinea-pig.



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